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Genotypic and Phenotypic Assays to Improve Strain Coverage Assessments of Sub-Capsular Meningococcal Vaccines

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Collaborating Establishment:

Meningococcal Reference Unit

Public Health England

Manchester Royal Infirmary

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Abbreviations

BBB	Blood-brain barrier
BIGSdb	Bacterial Isolate Sequence Database
bp	Base pair(s)
BSA	Bovine Serum Albumin
BSA/PBS	1% (w/v) Bovine Serum Albumin in Phosphate-buffered Saline
CBA	Columbia blood agar
CC	Clonal complex
CI	Confidence intervals
CSF	Cerebro-spinal fluid
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
ELISA	Enzyme-linked immunosorbent assay
fH	Complement Factor H
fHbp	Factor H-binding protein
GC	Gonococcal (broth or agar)
GCK	Gonococcal broth with 1% Kelloggs supplement and 1% NaHCO ₃
hSBA	Serum bactericidal antibody assay (using human-derived complement)
IMD	Invasive meningococcal disease
LOS	Lipooligosaccharide
MAC	Membrane attack complex
MATS	Meningococcal Antigen Typing System
Mbp	Mega base pair (million bp)
MEASURE	Meningococcal Antigen Surface Expression (assay)
MFI	Mean fluorescence intensity
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
ML	Molecular ladder
MRF MGL	Meningitis Research Foundation's Meningococcal Genome Library
MSC	Microbiological safety cabinet (Class I)
NadA	<i>Neisseria</i> adhesin A
NCBI	National Center for Biotechnology Information
NHBA	Neisserial heparin binding antigen
OD	Optical density
OMV	Outer membrane vesicle
Opa	Opacity-associated protein A
OPA	Opsonophagocytic activity
Opc	Opacity-associated protein C
PATH	Programme for Appropriate Technology in Health
PBS	Phosphate-buffered saline
PBT	Positive bactericidal threshold
PCR	Polymerase chain reaction
PE	Phycoerythrin
PFA	Paraformaldehyde
PFA/PBS	1% v/v Paraformaldehyde in Phosphate-buffered saline

PHE MRU	Public Health England's Meningococcal Reference Unit
PMN	Polymorphonuclear leukocyte
PV	Phase variation
rMLST	Ribosomal multilocus sequence typing
RP	Relative potency
RT	Room temperature (18-25 °C)
RV	Routine vaccinations
rSBA	Serum bactericidal antibody assay (using rabbit-derived complement)
SBA	Serum bactericidal antibody
SLV	Single-locus variant
ST	Sequence type
SWGA	Selective whole genome amplification
TBE	Tris-Borate EDTA
tfp	Type IV pilus/pili
UCL	University College London
VR	Variable region
WGS	Whole genome sequencing

Abstract

The licensure of recombinant protein-based meningococcal vaccines has increased the complexity of strain coverage assessments. In 2015, the 4CMenB vaccine was introduced into the UK national infant immunisation schedule and an Enhanced Surveillance programme was launched by Public Health England's Meningococcal Reference Unit. Meningococcal isolates, representing ~50% of laboratory-confirmed cases, are comprehensively characterised using whole genome sequencing and 4CMenB strain coverage is assessed phenotypically using the Meningococcal Antigen Typing System. For the remaining cases, which are confirmed using PCR only, strain characterisation was until recently restricted to geno-grouping and geno-subtyping. The purpose of this research was to establish new genotypic assays to improve strain coverage assessment among non-culture cases, as well as introduce the MEASURE assay to predict coverage of a second sub-capsular vaccine, rLP2086, among isolates. A PCR sequencing assay targeting the Factor H-Binding Protein antigen gene (*fHbp*) was developed and had an estimated analytical sensitivity limit of between 600ag/μL and 6fg/μL. Using this assay, *fHbp* was successfully sequenced from 1510 of the 1661 PCR-positive clinical samples tested (91%). The distributions of fHbp peptide variants among culture and non-culture strains were compared and, whilst differences were observed for a small number of predominant variants, the distribution was very similar within each capsular group.

The prospect of performing WGS directly from non-culture specimens was investigated using the Agilent SureSelectXT system. Eight of ten clinical specimens yielded genomes of acceptable quality. It was estimated that up to 54% of non-culture cases could be sequenced using this technique, however, the financial cost is currently prohibitive. Finally, the MEASURE assay was risk assessed and overnight formaldehyde incubation was introduced to ensure cells were fully fixed. The assay results were similar to those generated in a collaborating laboratory, however, further standardisation may be required.

These assays will help to increase the accuracy of strain coverage predictions of the currently-licensed and future sub-capsular meningococcal vaccines.

Declarations

This research was sponsored by Pfizer Vaccine Research (objectives 1, 2 and 4) and GlaxoSmithKline (objective 3).

Except where stated otherwise by reference or acknowledgment, the results presented herein are entirely my own. No part of this work has been submitted in support of candidature for any other degree or diploma.

Stephen Clark

Publications arising from this work

Much of the following work has been published in the following peer-reviewed articles:

- Clark, S. A., Lucidarme, J., Newbold, L. S. and Borrow, R. (2014) ‘Genotypic Analysis of Meningococcal Factor H-Binding Protein from Non-Culture Clinical Specimens.’ *PLoS ONE*, 9(2) p. e89921.
- Clark, S. A., Lekshmi, A., Lucidarme, J., Hao, L., Tsao, H., Lee-Jones, L., Jansen, K. U., Newbold, L. S., Anderson, A. S. and Borrow, R. (2016) ‘Differences between culture & non-culture confirmed invasive meningococci with a focus on factor H-binding protein distribution.’ *Journal of Infection*. 73(1) pp. 63–70.
- Clark, S. A., Doyle, R., Lucidarme, J., Borrow, R. and Breuer, J. (2017) ‘Targeted DNA enrichment and whole genome sequencing of *Neisseria meningitidis* directly from clinical specimens.’ *International Journal of Medical Microbiology*. 308 (2), pp.256–262.

Some of the following passages may bear minor resemblances to text within these articles. Excepting minor corrections/suggestions from co-authors, each of these articles was written solely by me and all words used are my own.

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1.0 BACKGROUND

1.1 Invasive Meningococcal Disease

1.1.1 Meningococcal Disease in the Pre-Vaccine Era

Invasive Meningococcal Disease (IMD) is an umbrella term used to describe the many maladies that can occur as a result of infection by the bacterial pathogen *Neisseria meningitidis*, also referred to as the meningococcus. Accounts of outbreaks resembling meningococcal infections date back to the late 17th century (Tyler, 2010). Vivid descriptions of outbreaks in 1806 in Geneva and 1806-1810 in the towns of New England illustrate the fear and confusion caused by the fulminant and deadly symptoms (Vieusseux, 1805; North, 1980; Tyler, 2010). In an 1810 review of local epidemics of the deadly ‘spotted fever’, Nathan Strong, a physician from Hartford, Connecticut, described cases of “a violent delirium” along with head pain and dark purple spots that would not recede under pressure. Writing of the disease, which peaked in the winter and spring months, Strong confessed to being “ignorant of its nature, mode of attack, or the weapons with which it might be most successfully combated” (Strong, 1810).

The meningococcus was first isolated and described in a Viennese laboratory in 1887. A pathologist named Anton Weichselbaum grew the bacterium in pure culture from six cases of epidemic cerebo-spinal meningitis. Weichselbaum had named the organism *Diplococcus intracellularis* to reflect the appearance and location of the bacterium upon initial observation (Weichselbaum, 1887). In 1891, Quincke developed the lumbar puncture technique which facilitated the isolation of the meningococcus from cerebro-spinal fluid (CSF) (Tyler, 2010).

In the first decade of the twentieth century, epidemics in Great Britain, the US and Canada featured fatality rates as high as 70-90% (Flexner, 1913). The work of pathologists such as George Jochmann and Simon Flexner led to the development of serum therapies. Flexner, a pathologist based in New York, studied the progression of the disease in animal models and developed antiserums by inoculating horses with

meningococcal cultures. After introduction of his antisera in 1907, fatality rates among the treated were reduced to 20-45% (Flexner, 1913).

Following the onset of the First World War in 1914, outbreaks in meningococcal disease were common place among the army camps of all countries. Consequently, the use of serum therapies increased in popularity. In 1916, Harold Amoss developed an agglutination method for the standardisation of meningococcal anti-serum (Amoss and Wollstein, 1916). It was from this early work that the first meningococcal typing systems arose, which subsequently led to the development of the first polysaccharide-based vaccines (Branham, 1953; Vipond et al., 2012).

The development of Sulphonamides in the 1930's also helped to reduce fatality rates, particularly during the Second World War. Resistance to Sulphadiazine first emerged in the early 1960's and led to the wide-spread adoption of beta-lactam antibiotics for treatment of IMD (Millar et al., 1963).

In 1923, the first edition of Bergeys Manual of Determinative Bacteriology was published and referred to the organism as *Neisseria intracellularis*. Whilst other binomials were used, this was the most commonly-used name for many years. In 1948, the sixth edition of Bergey's Manual used *Neisseria meningitidis* as the preferred name and, in 1963, the Judicial Commission of the International Committee on Bacteriological Nomenclature unanimously accepted *Neisseria meningitidis* as the official binomial for the meningococcus (Breed et al., 1948; International Committee on Bacteriological Nomenclature, 1963).

1.1.2 Clinical Features

The most common clinical presentation of IMD is rapid onset meningitis which occurs in around 60-70% of cases (European Centre for Disease Prevention and Control, 2012). Meningitis is defined as an infection of the meninges, the fine membranes that surround the brain and spinal column. As the bacterium penetrates the blood-brain barrier (BBB), it proliferates in the CSF eliciting damaging inflammatory responses. Initial symptoms vary widely from case to case, however, patients typically present with one or more of the following: headache, fever, nausea/vomiting, joint stiffness, light

sensitivity and confusion. Infants may also exhibit lethargy, irritability, poor feeding and raised fontanelles (Hart and Thomson, 2006; Brouwer et al., 2010). Perhaps one of the most characteristic aspects of the disease is the speed at which it can progress. Once invasion has occurred, bacterial load can increase rapidly and in many cases the patient's condition can become critical within hours of the onset of the initial symptoms (Hackett et al., 2002b).

Meningococcal septicemia, or meningococcaemia, can occur as the primary IMD manifestation (~ 30% of cases) or in conjunction with meningitis (~20% of cases) (European Centre for Disease Prevention and Control, 2012). The proliferation of bacteria within the bloodstream in many cases results in septicaemic shock characterised by severe inflammatory reactions leading to vascular damage and circulatory shutdown, often within 24 hours. In the first 12 hours of disease progression, general septicaemic symptoms such as cold, painful limbs and abnormal skin colouration may be observed along with a non-blanching petechial rash. If untreated, the disease can progress to disseminated intravascular coagulation manifested as *purpura fulminans*, severe cutaneous haemorrhaging and skin/limb necrosis (Tzeng and Stephens, 2000; Hart and Thomson, 2006; Pace and Pollard, 2012; Strelow and Vidal, 2013).

The activation of host inflammatory factors can result in vascular injury and damage to the cerebral tissues, leaving survivors with limb loss, deafness, seizures and motor and/or cognitive deficits, among other sequelae (Weber and Tuomanen, 2007; Karve et al., 2011). A 2010 meta-analysis of 132 studies found there to be a 9.5% median risk of at least one sequela following recovery from meningococcal meningitis (Edmond et al., 2010). Other less common forms of IMD include pneumonia, conjunctivitis, pericarditis and septic arthritis, often developing after meningitic/bacteraemic episodes (Stephens et al., 2007).

Fatality rates can vary substantially and disease outcome is often influenced by a range of factors such as the age of the patient, strain virulence, host genetic polymorphisms as well as the timing of symptom recognition and antimicrobial treatment. In developed countries, case fatality rates of between 5-10% are commonly reported

(Hahné et al., 2006; Gray et al., 2006; Trotter et al., 2007b; Ladhani et al., 2012b; Baccarini et al., 2013; Sadarangani et al., 2015). This figure, however, may be much higher in resource-poor settings or during outbreaks (Rouaud et al., 2006; Smith et al., 2006; Ceyhan et al., 2012; Strelow and Vidal, 2013).

1.1.3 Laboratory Diagnosis

Confirmation of suspected IMD cases is typically achieved through the isolation of *N. meningitidis* from an otherwise sterile bodily site. Isolates are commonly obtained from the blood and/or CSF, using venipuncture and/or lumbar puncture, respectively. Blood culture bottles are usually supplemented with culture medium and incubated at 35-37 °C (with 5% CO₂). Samples are subsequently sub-cultured onto agar and streaked for single colony isolation (World Health Organization, 2011).

N. meningitidis can grow on a variety of media, however, blood (sheep, horse or goat) or chocolate agars are typically used. The organism is non-haemolytic and, on blood agar, colonies appear round, grey and convex with a defined edge and a glistening surface. Meningococcal colonies exhibit a similar morphology when grown on chocolate agar; however they may appear slightly larger (World Health Organization, 2011).

Laboratories use a variety of different methods to confirm IMD depending upon the type of sample attained. For CSF samples, attempted culturing is often performed in parallel to cytological examination of the specimen. CSF turbidity and pleocytosis, especially increased numbers of polymorphonuclear leukocytes (PMNs), are indicative of bacterial meningitis. Gram staining of the CSF contents may help to identify bacterial cells within the sample. Meningococcal cells may be observed within PMNs and appear as 'coffee-bean'-shaped diplococci (World Health Organization, 2011)

Oxidase, catalase and carbohydrate utilization tests may be employed to differentiate isolated organisms from other common *Neisseria* species (World Health Organization, 2011). *N. meningitidis* is oxidase and catalase positive and can oxidise glucose and maltose, but not lactose and sucrose. Further phenotypic confirmation may be

provided using methods targeting meningococcal-specific antigens (Lucidarme et al., 2011b).

Many laboratories have developed real-time polymerase chain reaction (PCR)-based assays to amplify meningococcal deoxyribonucleic acid (DNA) directly from clinical specimens (Taha and Fox, 2007). These assays consist of DNA primers and probes specific to sequences within conserved meningococcal genes (e.g. *ctrA*, *sodC*, *crgA*) (Guiver et al., 2000; Thomas et al., 2011; Taha, 2000). This strategy allows laboratory confirmation without the need for *in vitro* culture of the organism. This is particularly invaluable as many specimens do not yield a culturable isolate (Heinsbroek et al., 2013).

1.1.4 Treatment

In the UK, urgent hospital admission of suspected bacterial meningitis or meningococcal septicaemia patients is recommended (National Institute for Health and Care Excellence, 2010). Those with a non-blanching rash (i.e. suspected septicaemia) or those who cannot be immediately hospitalised should be administered a single dose of parenteral benzylpenicillin, ideally intravenously, prior to admission. The dosage ranges from 300 mg to 1200 mg depending on patient age (National Institute for Health and Care Excellence, 2010).

Following admission, third generation cephalosporins, such as Ceftriaxone, are the preferred treatment. Cefotaxime plus either amoxicillin or ampicillin should be administered to children younger than 3 months of age (National Institute for Health and Care Excellence, 2010).

Due to the speed of disease progression, prompt antimicrobial therapy is important in suspected IMD cases. A 2006 meta-analysis found a positive association between pre-admission antimicrobial treatment and reduced mortality (Hahné et al., 2006) and further work has shown that delays in receiving such treatment are likely to influence case fatality rates (Proulx et al., 2005).

1.2 *Neisseria meningitidis*: Cellular Features and Virulence Factors

N. meningitidis is a non-motile, gram-negative, facultative anaerobe found within the class Betaproteobacteria. The organism is an exclusively human pathogen and the nasopharyngeal tract represents its primary reservoir (Stephens, 2009). The bacterium possesses many features that contribute to its survival within this niche and the potential for invasion of host tissues.

1.2.1 Polysaccharide capsule

One of the predominant features of the meningococcal cell is the polysaccharide capsule. The chemical composition of the polysaccharide capsule is the basis of the principal system of categorisation within the species: the Group. Traditionally referred to as serogroup, the classification system was developed throughout the twentieth century using serological methods (Craven et al., 1978). The term 'genogroup' may also be used when the determination has been achieved through genetic characterisation.

There are 12 different capsular groups (A, B, C, E, H, I, K, L, X, W, Y, Z) and of these, six cause the vast majority of disease (A, B, C, W, X and Y) (Harrison et al., 2009). The capsular polysaccharides of group B, C, W and Y contain sialic acid. Group B and C capsules are composed entirely of sialic acid with $\alpha 2 \rightarrow 8$ and $\alpha 2 \rightarrow 9$ linkages, respectively (Bhattacharjee et al., 1975). Group W and Y polysaccharides contain alternating units of D-galactose or D-glucose and sialic acid, respectively (Bhattacharjee et al., 1976). Group A and X are composed of repeating sub-units of N-acetyl-D- mannosamine-1-phosphate and N-acetylglucosamine 1-phosphate, respectively (Liu et al., 1971; Apicella and Robinson, 1972).

The genes responsible for capsular synthesis and expression (termed *cps*) are well characterised for all groups (Harrison et al., 2013). Despite significant differences in polysaccharide composition, the different loci are remarkably well conserved between these groups. All loci can be divided into six regions (A-D, D' and E) each containing two to nine genes. Region A contains the genes responsible for polysaccharide biosynthesis and constitutes the majority of the diversity between the different capsular groups. The fourth gene within this region (*cs*, *siaD*, *mynA*) encodes the polysialyltransferase,

which determines the capsular composition. The polysialyltransferase gene of group B and C strains exhibit ~60% similarity whilst ~98% homology is seen between this gene among group Y and W strains (Claus et al., 1997). The translated peptides of the group W and Y polymerases are consequently very similar and a single amino acid (310) determines the specificity to galactose or glucose, respectively (Claus et al., 2009). Due to these similarities, a small proportion of invasive strains express capsular polysaccharide composed of sialic acid with both D-glucose and D-galactose which, in some cases, cross-react with both anti-Y and anti-W sera (personal correspondence, Dr Steve Gray, PHE MRU).

Group A polysaccharides are naturally O-acetylated by the O-acetyltransferases encoded by the *csaC* gene (*mynC*). Groups C, W and Y polysaccharides, however, can be O-acetylated or non-O-acetylated at the sialic acid residues. In the UK, O-acetylation was seen among ≥70% of invasive group C and Y strains, whilst only a small proportion (8%) of group W strains are O-acetylated (Borrow et al., 2000; Longworth et al., 2002; Balmer et al., 2002). In group C organisms the O-acetyltransferase is encoded by the *cssE* gene (*oatC*) and in group W and Y organisms by *cssF* (*oatWY*) (Claus et al., 2004; Harrison et al., 2013).

Regions B and C contain genes involved in the transport and membrane translocation of the high-molecular weight polysaccharide. The remaining regions are involved in lipooligosaccharide (LOS) synthesis and sialylation (Harrison et al., 2013).

1.2.2 Porins

Of the many surface proteins expressed by meningococci, the major porins, PorA (class I) and PorB (class II or III), are the most abundant and both expressed by the majority of meningococcal strains (Feavers et al., 1996; Law et al., 2014). As with many gram-negative porins, both PorA and PorB exhibit a trimeric 16-stranded β-barrel peptide structure and allow the translocation of hydrophilic molecules in and out of the cell (Derrick et al., 1999). Although coded from a same locus within the genome, there is notable sequence variation between class II and class III PorB peptides (PorB2 and PorB3, respectively), with PorB3 much more closely related to porins of other *Neisseria* species (e.g. PorB1a of *Neisseria gonorrhoeae*) (Derrick et al., 1999).

Due to the abundance of PorA on the cell surface, it is one of the most immunodominant antigens with convalescent antibodies specific to exposed epitopes, particularly to loop IV (Van der Ley et al., 1991; Idänpään-Heikkilä et al., 1995). In encapsulated strains, PorA binds to the C4 binding protein (C4bp) complement inhibitor, increasing serum survival (Jarva et al., 2005). Similarly, PorB2 is able to bind the alternative complement pathway regulator Factor H (fH), reducing C3 deposition and serum susceptibility (Lewis et al., 2013).

1.2.3 Lipooligosaccharides

The LOS is a major feature component of the meningococcal cell membrane and is crucial to its structural integrity. The LOS maintain the negative charge of the membrane and is an important moderator of the host immune response (Unkmeir et al., 2002b). Meningococcal LOS is cytotoxic and can cause host endothelial monolayer dysfunction and cytoskeletal reorganisation (Slanina et al., 2011). The imbedded inner core structure principally consists of acylated Lipid A, whilst the outer portion contains variable α and β oligosaccharide chains (Kahler and Stephens, 1998). Lipid A is relatively well conserved between strains, however, inner core structural variation can significantly impact host immune response and disease progression. LOS with six fatty acyl chains on the Lipid A moiety (hexa-acylated) are strongly associated with invasive strains, in contrast to those with five acyl chains (penta-acylated), which exhibit decreased toll-like receptor 4-mediated induction of cytokines by macrophages (Fransen et al., 2010; Rodenburg et al., 2012; John et al., 2016). Structural modifications to the inner core (e.g. sialylation) also correlate with invasive potential and aid immune survival (Klein et al., 1996; Ram et al., 2003; Plant et al., 2006; Lewis et al., 2012; Unkmeir et al., 2002b), whilst the addition of phosphoethanolamine can increase adherence to host cells (Takahashi et al., 2008).

1.2.4 Pili

Pili are filamentous organelles composed of peptide polymers that protrude many micrometres from the cell surface. Pili are indispensable during initial epithelial attachment, however, the precise mechanisms of these interactions are not yet fully understood (Nassif et al., 1994; Pujol et al., 1997; Kirchner and Meyer, 2005; Exley et

al., 2009). *N. meningitidis* expresses Type IV pili (tfp), the principal structure of which consists of repeating PilE subunits arranged in a helical configuration around a hydrophobic core (Giltner et al., 2012). PilE undergoes extensive antigenic variation through positive immune selection and inter-genomic recombination mediated by multiple silent homologous genes (*pilS*) (Andrews and Gojobori, 2004). Whilst this variation can influence interactions with host cell receptors, PilC, located at the pilin tip, also appears to play a key role in the epithelial cell adherence (Nassif et al., 1994; Rahman et al., 1997; Källström et al., 1998; Morand et al., 2009). Additionally, the minor pilin PilX plays an essential role in meningococcal aggregation during colonisation (Hélaine et al., 2005).

1.2.5 Opacity proteins

The Opacity proteins Opc and Opa are surface exposed adhesins that mediate binding to and invasion of host epithelial and endothelial cells (Virji et al., 1993; de Vries et al., 1996; Muenzner et al., 2000; Johswich et al., 2013) as well as interactions with phagocytic cells (McNeil and Virji, 1997). Opc expression can also aid serum resistance through the binding of vitronectin, a repressor of the late complement pathway (Griffiths et al., 2011; Hubert et al., 2012).

1.2.6 IgA protease

The IgA protease autotransporter can be an important determinant of invasive potential by cleaving the heavy chain of IgA1 immunoglobulins preventing antibody-mediated clearance (Plaut et al., 1975). In many hyper-invasive strains (e.g. ST-11 complex), these Type V autotransporters feature specific nuclear localisation signals, which facilitate entry into the nucleus of epithelial cells and cleavage of the NF- κ B transcriptional regulator resulting in apoptosis of the host cell (Besbes et al., 2015). Furthermore, IgA protease-mediated cleavage of LAMP1, a major component of lysosomes, promotes intracellular survival of invasive strains (Lin et al., 1997; Ayala et al., 2001).

1.2.7 Other virulence factors

Within the host, the sequestration of extra-cellular iron, usually in the form of Transferrin, Lactoferrin or Haemoglobin/Haptoglobin, is achieved via specific sets of cognate receptors (i.e. TbpAB, LbpAB, HpuAB and HmbR), which are important for survival and virulence *in vivo* (Perkins-Balding et al., 2004; Renauld-Mongenie et al., 2004; Lucidarme et al., 2013b).

Other potential virulence factors include NalP, a phase variable autotransporter which is involved in cleavage of host complement/meningococcal outer membrane proteins and plays an important role in biofilm formation (Roussel-Jazédé et al., 2010; Del Tordello et al., 2014; Perez-Ortega et al., 2017), Meningococcal surface fibril (also termed NhhA) which mediates adhesion and down regulation of the host complement cascade (Griffiths et al., 2011), and the adhesins MspA and App (Hill et al., 2010).

1.2.8 The meningococcal genome

The meningococcal genome is approximately 2.2×10^6 base pairs (bp) in length, organised in a typical circular chromosome (Parkhill et al., 2000). *N. meningitidis* is naturally transformable, readily receiving exogenous DNA from its environment. There is strong genomic evidence of horizontal exchange, incorporating DNA from other nasopharyngeal neisserial species such as *N. lactamica* (Linz et al., 2000; Lucidarme et al., 2011a), as well as other co-habiting genera such as *Haemophilus* (Kroll et al., 1998). This transformation is made more efficient by the presence of a 10-12 base pairs (bp) DNA uptake sequence, observed at over 2000 loci within the genome (Treangen et al., 2008). As a result, meningococcal genomes typically feature many artefacts of recombination, which represents a primary driver of diversity within the species (Jolley et al., 2005).

Many important meningococcal virulence genes are subject to phase variation (PV), allowing changes in their expression. This is achieved through slipped-strand mispairing of homopolymeric nucleotide tracts either within the open-reading frame or in the flanking gene promoter affecting transcription and/or translation (Sarkari et al., 1994; Berrington et al., 2002; Oldfield et al., 2013; Lucidarme et al., 2013b). This selective

control of expression levels can facilitate colonisation or invasion of the host and avoidance of the immune responses (Tauseef et al., 2013; Alamro et al., 2014; de Vries et al., 1996; Hubert et al., 2012). Transformation of heterologous neisserial DNA has been found to increase PV frequency (Alexander et al., 2004) and there is evidence that PV frequency and general mutability is influenced by the functionality of the mismatch repair enzyme DNA adenine methyltransferase (dam) (Bucci et al., 1999). A similar study, however, found no such link but mismatch repair proteins *MutS* and *MutL* were shown to be important regulators of PV frequencies (Richardson and Stojiljkovic, 2001; Colicchio et al., 2006).

1.3 Colonisation and Carriage

1.3.1 Mechanisms of Colonisation

Transmission of the meningococcus occurs through direct contact with oral or nasal secretions or inhalation of air-borne droplets (Tzeng and Stephens, 2000). The colonisation of the nasopharynx begins with the aggregation of the bacterial cells and subsequent adherence to nasopharyngeal tissues, particularly non-ciliated columnar cells. Both of these actions are facilitated by tfp through inter-bacterial PilX:PilX interactions and PilC-mediated binding to host receptors (such as CD46), respectively (Stephens et al., 1983; Read et al., 1995; Källström et al., 1998; Hélaine et al., 2005).

The attachment of tfp is followed by the formation of microcolonies on the apical surface leading to close interactions between outer membrane adhesins (e.g. Opc, Opa, TspA, NadA) and host cell receptors such as CD66a (Billker et al., 2000; Comanducci et al., 2002; Oldfield et al., 2007; Virji et al., 1993; Johswich et al., 2013). The expression of sialic acid-based capsules can, however, mask these adhesins and reduce epithelial attachment (Stephens et al., 1993; Hammerschmidt et al., 1996; Bartley et al., 2013). Furthermore, the glycosylation of pili can lead to detachment of meningococcal cells, facilitating the colonisation of neighbouring tissues (Chamot-Rooke et al., 2011). Recent work also suggests that a filamentous bacteriophage, MDAΦ, promotes aggregation and compensates for the lack of piliation in the upper layers of the adherent biomass (Bille et al., 2017)

The formation of meningococcal biofilms on respiratory epithelial cells has been demonstrated *in vitro* using both encapsulated and unencapsulated strains (Neil et al., 2009). The utilisation of extracellular DNA, which is important for the stability of biofilms of other bacterial species, can vary between different virulent meningococcal strains. This has been suggested as a possible explanation for observed differences in carriage rates between hyper-invasive lineages (Lappann et al., 2010).

1.3.2 Transmission and Carriage in the Population

Carriage of the organism can be transient, with colonisation lasting a matter of days, or can persist for several months (Stephens, 2009). In endemic populations, a consistent carriage profile can be seen with relatively low carriage rates observed in children under 4 years (<5%). The rate of carriage slowly increases with the population age before rising sharply from 15 years of age and peaking at around 19 years, an age group in which carriage rates of ~25-30% are frequently observed (Cartwright et al., 1987; Caugant et al., 1994; MacLennan et al., 2006; Christensen et al., 2010; Jeppesen et al., 2015). The rise of carriage in adolescence is generally attributed to attendance of closed or semi closed institutions such universities and military establishments where close contact with others is increased. A study of UK university students suggested that specific social behaviours (e.g. smoking, kissing, visiting pubs/clubs) largely account for the sharp increase in carriage from 15 years onwards (MacLennan et al., 2006). Into adulthood the carriage rate reduces and plateaus at ~10% (Cartwright et al., 1987; Caugant et al., 1994; Christensen et al., 2010).

Studies of meningococcal carriage in sub-Saharan African populations report more discordant carriage profiles with rates between 3% and 30% reported across the region (Trotter and Greenwood, 2007; MenAfriCar Consortium, 2015; Basta et al., 2017). In recent surveys, higher carriage rates were reported in children (5-14 years) than in adolescents/young adults (15-29 years) (Diallo et al., 2016; MenAfriCar Consortium, 2015). This variation may reflect differences in social norms and behaviours (e.g. lower higher education attendance) between countries within this region and those of other parts of the world.

The characteristics of the carried strains also influence the level of carriage. Marked differences in the carriage prevalence of strains of different capsular groups have been previously observed (Maiden and Stuart, 2002; Maiden et al., 2008). During the UK group C epidemic of the late 1990's, surprisingly low carriage of group C strains were observed including the causative lineage 11.2 (ST-11 complex) strain. This was despite high disease incidence and higher carriage of group B, W and Y strains (Jones et al., 2000; Maiden et al., 2008; Maiden and Stuart, 2002).

1.4 Pathogenesis

1.4.1 Traversing the Epithelium

Colonisation may in some cases lead to migration of meningococci through the pharyngeal epithelium. There is some evidence to suggest that environmental factors such as smoking and dry, dusty conditions may facilitate meningococcal invasion by weakening the pharyngeal lining (Sultan et al., 2005; Stanwell-Smith et al., 1994). Much of the evidence suggests meningococci migrate using a transcellular route beginning with the tfp-dependent formation of plaques on apical surface (Merz et al., 1999; Sutherland et al., 2010; Pujol et al., 1997) followed by subversion of intracellular trafficking systems and manipulation of the epithelial cytoskeleton leading to the internalisation of the meningococcal cells (Barrile et al., 2015; de Vries et al., 1996; Pujol et al., 1997). An alternate invasion mechanism in which internalised pathogenic *Neisseria* spp. "hitchhike" across the epithelium within PMNs has been demonstrated *in vitro* (Söderholm et al., 2011).

Upon entry into the host bloodstream, rapid multiplication is facilitated by the modified expression of around 30% of genes including immune regulators and nutrient uptake systems (Echenique-Rivera et al., 2011). Infection can infrequently result in a low level, chronic bacteraemia presenting with limited but prolonged symptomologies (Thimmesch et al., 2016). Chronic meningococcaemia is probably a result of strong host immunity and/or attenuated strain virulence determinants such as lower LOS reactivity (Prins et al., 1998; Brouwer et al., 2011). In many cases, however, proliferation of the invading strain causes a rapid increase in LOS triggering the release of pro-inflammatory cytokines which can lead to acute septic shock (Brandtzaeg et al., 1989).

Although uncommon, meningococci can be seeded from the bloodstream into compartmentalised spaces causing pericarditis and septic arthritis, among other systemic infections (Dulović et al., 2009).

1.4.2 Traversing the BBB

The BBB is principally composed of junctional complexes located between endothelial cells, preventing the paracellular passage of hydrophilic macromolecules, neurotoxic compounds and foreign agents (Coureuil et al., 2012). The meningococci appear to use both transcellular and paracellular routes to cross the BBB and enter the subarachnoid spaces. The tfp play a crucial role in adherence to endothelial cells and the formation of cortical plaques, which can result organisational changes in the endothelial actin filaments and subsequent internalisation (Eugène et al., 2002; Mikaty et al., 2009; Lécuyer et al., 2012; Takahashi et al., 2012). tfp also elicit relocalisation of junctional proteins resulting in leakage and allowing the paracellular transport of meningococcal cells into the subarachnoid space (Coureuil et al., 2009).

In the CSF, the bacteria multiply at an enhanced rate owing to the dearth of complement proteins and immunoglobulins. Interactions between the invading strains and the cells of the leptomeninges elicit the release of a medley of pro-inflammatory cytokines including interleukin-6 and interleukin-8. *In vitro* data suggests the presence of meningococcal components such as tfp, capsular polysaccharide and LOS may influence the nature of the inflammatory response (Christodoulides et al., 2002).

1.5 Immunity

1.5.1 Innate Immunity and the Complement System

The innate immune system consists of phagocytic cells and a host of cationic peptides with a large array of anti-microbial functions. The cytotoxic peptide Bactericidal/Permeability-Increasing protein, for example, is released from granulocytes and possesses endotoxin-neutralising activity (Schultz and Weiss, 2007). The cathelicidin LL-37, expressed by immune and epithelial cells can also kill meningococci through membrane-destabilising surface interactions (Jones et al., 2009)

Consisting of over 30 plasma proteins, the complement system plays a crucial role in recognition and elimination of the invading meningococcal cells. Three distinct proteolytic pathways culminate in the formation of C3b which covalently binds to the meningococcal membrane surface (Lewis and Ram, 2014). C3b has a dual function in relation to meningococcal clearance. Firstly, it serves as an effective opsonin, promoting phagocytosis of the invading cell by proximate macrophages and PMNs (Ehlenberger and Nussenzweig, 1977). In addition to its opsonic properties, the binding of C3b leads the continuation of the proteolytic complement cascade and the eventual formation of the membrane attack complex (MAC), a multi-protein complex imbedded within the membrane of the invading cell. The MAC acts as a pore, destabilising the osmotic balance and causing cell lysis (Lewis and Ram, 2014).

The Classical complement pathway and Lectin complement pathway are activated by membrane-bound antigen-specific antibody (IgG or IgM) or carbohydrate-specific mannose binding lectin, respectively. Conversely, the Alternate complement pathway is not target-specific but is activated spontaneously at a low level on all membrane surfaces. Within host tissues, however, this spontaneous activation is inhibited by a number of negative pathway regulators (e.g. Factor H) (Lewis and Ram, 2014).

The critical importance of MAC-mediated lysis is evidenced by increased disease incidence in patients with late complement component deficiencies (Fijen et al., 1999; Wright et al., 2009; Kuijpers et al., 2010) and those taking complement inhibitors (Struijk et al., 2013). These findings also suggest that opsonophagocytic activity (OPA) alone is not sufficient for immune protection. The contribution of OPA in the prevention of IMD is not yet fully understood. Limited laboratory data suggests that complement-mediated phagocytosis may be an effective method of killing, at least against some meningococcal strains (Ross et al., 1987). Additionally, the increased incidence of meningococcal disease in asplenic individuals indicates a significant contributory role as most opsonised bacteria are cleared by phagocytic macrophages in the spleen (Ram et al., 2010).

Capsular polysaccharide plays an important role in the organism's defence against host innate immune response. Expression of homopolymeric sialic acid capsules (e.g. groups

B and C) reduces C3 deposition on the bacterial surface thus inhibiting alternative pathway-mediated lysis (Jarvis and Vedros, 1987; Vogel et al., 1997; Uria et al., 2008; Agarwal et al., 2014). Conversely, data generated using isogenic strains suggests that the chemical composition of group W and Y capsules may actually promote rapid activation of C3a and deposition of C3 (Ram et al., 2011). Capsular expression can also reduce deposition of LL-37, reduce adherence to dendritic cells thus impeding phagocytosis, and the expression of group C polysaccharide can inhibit LOS-triggered cytokine release by binding to CD14 of human monocytes (Unkmeir et al., 2002a; Kocabas et al., 2007; Jones et al., 2009).

1.5.2 Acquired Immunity and Serum Bactericidal Antibody (SBA) Activity

Although the innate immune response represents the primary process of meningococcal clearance, protective immunity against *N. meningitidis* is dependent upon the adaptive immune system, in particular the presence of circulating serum bactericidal antibodies (SBA) against the infecting strain (Erlach and Congeni, 2012). These antibodies elicit a rapid response, providing opsonic stimulation of phagocytes and activation of the classical complement pathway.

The influential work by Goldschneider, Gotschlich and Artenstein in the 1960's identified SBA to be the key protective factor against meningococcal disease using *in vitro* bactericidal assays to quantify SBA activity (Goldschneider et al., 1969a). Within US army camps, only 5.6% of recruits who developed group C meningococcal disease exhibited SBA activity against the epidemic strain, compared to 82% of controls who didn't develop disease. A disease incidence of 38.5% was observed amongst those with no bactericidal activity against the acquired group C strain, compared to 1% of the total population (Goldschneider et al., 1969a). This work also revealed an inverse correlation between the numbers of participants with SBA activity and the incidence of meningococcal disease across different age groups.

A sharp reduction in SBA activity is seen shortly after birth (within the first six months) due the depletion of maternal antibodies. SBA activity then gradually increases with age, rising sharply throughout adolescence in particular (Trotter et al., 2003, 2007a). The gradual increase in SBA activity is attributed to the increased acquisition of carriage

as age increases and supports initial evidence that carriage of meningococci or other *Neisseria* commensals (e.g. *N. lactamica*) has an immunising effect (Goldschneider et al., 1969b; Cartwright et al., 1987; Christensen et al., 2010). Despite this pattern, a clearly-defined inverse correlation between SBA activity and IMD incidence is not always observed (Trotter et al., 2012, 2013), prompting suggestions that other immune mechanisms (e.g. OPA) may be partly responsible for the dramatic decrease in cases as age increases (Granoff 2009).

SBA activity, as measured using titres derived from *in vitro* SBA assays, is the accepted surrogate of protection against IMD. Using surrogates provides a means by which the level of protection within a population can be reliably predicted and allows the assessment of vaccine efficacy without the need for population-wide efficacy studies, which are costly and difficult to carry out due to relatively low IMD incidence (World Health Organization, 1976; Borrow et al., 2005; Frasch et al., 2009).

An SBA titre is defined as the serum dilution at which 50% of the bacterial supplement is killed during the bactericidal assay. Complement within test sera is heat-inactivated prior to introduction of exogenous complement within non-immune serum. This equalises the concentrations of complement across the dilution series and ensures titre differences between test sera are due to differences in bactericidal antibody concentrations only (McIntosh et al., 2015). The aforementioned work by Goldscheider *et al* established a group C protective SBA titre of ≥ 4 when using exogenous human complement in the bactericidal assay (hSBA). This hSBA cut-off has been shown to reliably correlate with protection against group B and C disease in subsequent polysaccharide and outer membrane vesicle (OMV) vaccine efficacy trials (Artenstein et al., 1970b; Holst et al., 2003). The lack of standardisation of human complement sources has, however, led to the adoption of rabbit complement in SBA assays (rSBA) (Maslanka et al., 1997; Borrow et al., 2005). The use of rabbit complement leads to significantly higher titres than those obtained using human complement, especially against group B strains (Zollinger and Mandrell, 1983). This effect was observed during group C conjugate polysaccharide vaccine trials in the 1990s (Borrow et al., 2001). The difference is thought to be at least partly due to the inability of *N. meningitidis* to bind rabbit-derived complement fH. Conversely, binding of human fH in the hSBA leads to

down-regulation of the alternative complement pathway and comparatively lower SBA titres (Granoff et al., 2009).

Following the introduction of group C conjugate vaccines in the UK in 1999, a validation study using post-licensure sera was performed which took a population-based approach and proposed a protective rSBA correlate of $\geq 1:8$ for group C disease (Andrews et al., 2003).

Other functional assays such as whole blood killing assays and OPA assays have been developed, however, assay protocols are not yet fully standardised and, unlike the SBA assay, the relationship between these assays and protective immunity on a population level is yet to be fully understood (Vermont and Van Den Dobbelsteen, 2002; Findlow et al., 2006; Welsch and Granoff, 2007; Humphries et al., 2015). In addition to functional assays, enzyme-linked immunosorbent assays (ELISA) and multiplex bead-based immunoassays are commonly used in trials to measure total antigen-specific antibody concentrations (Laher et al., 2006; Vermont and Van Den Dobbelsteen, 2002).

1.6 Typing and Surveillance

1.6.1 Grouping

Laboratory determination of the capsular group was traditionally achieved using serum agglutination techniques, however, targeted immunoassays (e.g. the 'dot-blot' ELISA) utilising capsular polysaccharide-specific murine monoclonal antibodies have been adopted as the standard phenotypic method (Wedge and Rosenqvist, 1990; Gray et al., 2006). Capsular group can be determined genotypically through real-time PCR detection and/or sequencing of the *siaD* gene. This can be particularly useful in the absence of a clinical isolate (Heinsbroek et al., 2013).

1.6.2 Typing and Subtyping

As immunologically-dominant surface antigens, characterisation of PorA and PorB form an important part of meningococcal typing systems; with variation in these proteins distinguishing the Subtype and Type of different strains, respectively (Jolley et al., 2007). Much of the sequence variation within the different porin classes is restricted to

eight surface-exposed loops, a likely result of immune selective pressures during host colonisation (Smith et al., 1995). Typing of these antigens has traditionally been achieved with panels of antibodies that recognise specific surface-exposed epitopes, or variable regions (VR), using co-agglutination or dot blot ELISA (Wedeg and Rosenqvist, 1990). PorA VRs are located at the apex of loops I and IV (VR1 and VR2, respectively), which are characterised individually (Van der Ley et al., 1991). VR3 (located at loop V) is occasionally characterised, however, it offers limited additional discriminatory power. The strain Type is determined by collective detection of four PorB VRs located on loops I, V, VI and VII (Sacchi et al., 1998). The lack of standardisation of serological typing reagents led to the development of genotyping assays to sequence the coding regions of the porin VRs (McGuinness et al., 1993; Sacchi et al., 1998).

1.6.3 Immunotyping

Structural variation in the oligosaccharide chains of the LOS forms the basis of Immunotyping scheme. Unique oligosaccharide chain compositions are assigned Immunotypes (L1 to L12) and can influence host cell adhesion, serum survival and immune interactions (Plant et al., 2006; Hubert et al., 2012). The L3,7,9 immunotype is strongly associated with group B invasive strains, whilst L1,8,10 is associated with carriage strains (Jones et al., 1992).

1.6.4 Population Typing

Developed in the mid-1980s, Multi-locus Enzyme Electrophoresis (MLEE) provides the means of elucidating the underlying population structure of invasive meningococcal strains (Selander et al., 1986). The technique identifies differences in the electrophoretic migration of seven evolutionarily-restricted 'house-keeping' enzymes between meningococcal strains. These differences correlate with variation in the enzymes amino acid structure and, therefore, genetic variation in the encoding genes. Unique electrophoretic patterns produced on an acrylamide gels were assigned arbitrary numeric identifiers and strains producing the same/similar patterns were grouped into electrophoretic types, which represent closely-related meningococcal lineages.

For the first time, MLEE allowed researchers to elucidate the diversification of meningococcal strains on a longer evolutionary timescale to those observed when studying elements under selection pressure (i.e. surface antigens) (Caugant et al., 1986; Selander et al., 1986). Despite efforts at standardizing the MLEE assay, differences in protocols and reagents led to poor reproducibility between reference laboratories. These difficulties, combined with reductions in the cost of PCR and DNA sequencing in the 1990's, led to the transition to Multilocus Sequence Typing (MLST) (Maiden et al., 1998). MLST involves sequencing fragments of the genes encoding the seven MLEE enzymes. Numeric IDs are assigned to unique nucleotide alleles eliminating the ambiguity observed with MLEE. Unique combinations of the seven allelic IDs are given numeric sequence type (ST) IDs. STs can then be clustered together (typically around a central, ancestral ST) with others STs featuring the same allelic variants at four or more of the seven loci. These groups of evolutionarily similar strains are termed clonal complexes (CC). The MLST scheme for meningococci, as well as many other bacterial pathogens, is currently coordinated from online databases hosted by University of Oxford, England (www.pubMLST.org). Despite the assignment of over 13,000 unique meningococcal MLST STs, many of the IMD cases reported globally are caused by strains belonging to a limited number of 'hyper-virulent' CCs (Caugant and Maiden, 2009).

1.6.5 Whole Genome Analysis

Shortly after the development of MLST, whole genome sequencing of the first two meningococcal genomes MC58 (group B) and Z2491 (group A) was completed (Tettelin et al., 2000; Parkhill et al., 2000). Subsequent advancements in massively parallel sequencing technologies have allowed cost-effective sequencing of bacterial genomes within practical time frames (Bertelli and Greub, 2013).

This wealth of information has enabled extensive epidemiological analyses of invasive sub-lineages, vaccine antigen distributions as well as detailed genomic comparisons of past and emerging invasive clones (Hill et al., 2015; Brehony et al., 2015; Lucidarme et al., 2015). The significant increase in the proportion of characterised genes has also vastly improved the resolution of MLST analyses and a number of extended MLST

schemes have been developed utilising varying numbers of selected loci (Maiden et al., 2013) (e.g. ribosomal MLST (rMLST) comprising 53 ribosomal protein subunit genes (Jolley et al., 2012) and core genome MLST schemes which include those genes purported to be present in all/most meningococcal strains (Bratcher et al., 2014)).

Despite the increasing use of this technology for analyses of a wide range of bacterial pathogens (Tagini and Greub, 2017), standard WGS protocols generally require high concentrations of purified target DNA template (typically 5-50 ng/μL). DNA enrichment techniques, which allow the isolation and/or amplification of specific DNA sequences within mixed samples (e.g. clinical specimens) have been developed and a number of commercial assays are available. These assays utilise different approaches such as degradation or removal of extra-cellular or methylated DNA, and/or selective target amplification (Hansen et al., 2009; Clarke et al., 2017). Enrichment assays such as the Agilent SureSelectXT and Roche NimbleGen SeqCap systems involve hybridisation or “capture” genomic fragment using target-specific oligonucleotide “baits” (García-García et al., 2016). These systems have been used successfully to perform WGS of a number of viral and bacterial targets from clinical specimens (Depledge et al., 2011; Christiansen et al., 2014; Brown et al., 2015, 2016).

1.7 Epidemiology

1.7.1 Group A

Throughout the early twentieth century, the majority of meningococcal disease in Europe and South America was caused by group A meningococci. During World War I, military camps throughout the Europe and America experienced sporadic group A outbreaks resulting in over 10,000 cases (Abio et al., 2013; Baccarini et al., 2013). In recent years, group A strains are only predominant in Africa and Asia (including parts of Russia) (Achtman et al., 2001; Teyssou and Muros-Le Rouzic, 2007; Harrison et al., 2009).

Since the 1960's, multiple pandemic waves of distinct group A sub-clones, mostly originating in East Asia, have spread into Europe, Africa and beyond (Teyssou and Muros-Le Rouzic, 2007). Large group A epidemics have repeatedly occurred across

many Asian countries (e.g. China, Mongolia, India, Pakistan and Nepal) throughout the past century with fatality rates varying from 7-33% (Vyse et al., 2011).

Historically, the highest IMD incidence, of any capsular group, was found in a band of countries of sub-Saharan Africa during the 'dry season' (November to May, Fig. 1-1). During this period, incidence of group A disease could be 10-100 fold greater than that of the corresponding wet season. This region, often referred to as 'the Meningitis Belt', experienced cyclic epidemics of group A disease every 5-12 years, with high fatality rates (Mueller and Gessner, 2010; Koutangni et al., 2015).

As well as persistent occurrences of disease by ST-1 complex and ST-4 complex sub-clones throughout the past 60 years, ST-5 complex strains have been imported into Africa several times, for example by Hajj pilgrims returning from Mecca in 1987, allowing the spread of this lineage to all corners of the continent (Guibourdenche et al., 1996; Caugant and Nicolas, 2007). In the 1990s, this ST-5 strain was largely replaced by an ST-7 sub-clone, which was subsequently replaced by descendant ST-2859 clones in several meningitis belt countries (Nicolas et al., 2001, 2005; Lamelas et al., 2014).

In 2010, the Meningitis Vaccine Project, a collaboration between the Serum Institute of India, Programme for Appropriate Technology in Health (PATH) and the World Health Organization, commenced the successful introduction of a group A conjugate vaccine (MenAfriVac) across the meningitis belt (Djingarey et al., 2015). The vaccine was administered to over >200 million individuals aged 1-29 years and has resulted in a manifest reduction of group A disease within vaccinated populations (Diomande et al., 2015). Long-term routine use of MenAfriVac into the Expanded Programme on Immunization is recommended in order to prevent the resurgence of group A disease in the meningitis belt (Karachaliou et al., 2015).

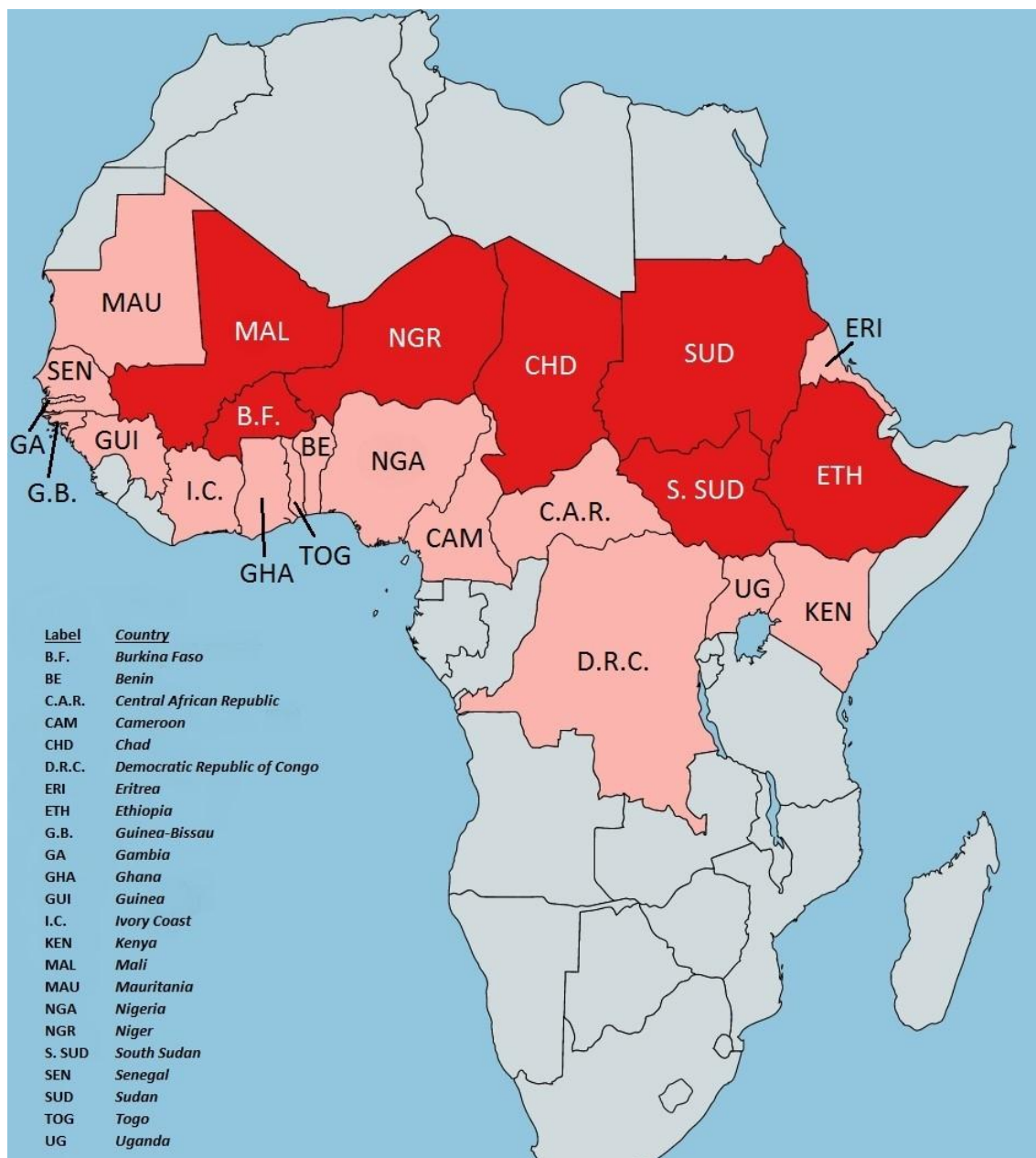


Figure 1-1: A map of Africa with The Meningitis Belt highlighted. The countries considered to be in the Meningitis Belt are coloured red and labelled. The countries coloured in dark red have historically experienced particularly high incidence (>10 cases/100,000). Map template sourced at yourfreetemplates.com.

1.7.2 Group C

Significant incidence of group C disease has historically been observed across Europe and the Americas (Baccarini et al., 2013). During the 1990s, a high incidence of group C disease was seen in the UK, Spain, Ireland and a number of other European nations, primarily driven by the expansion of a single hyper-virulent clone (C:2a:P1.5,2, ST-11). The incidence in many European countries increased dramatically from <1 to ~5 per 100,000 people (Trotter et al., 2007b; Bijlsma et al., 2014).

Group C conjugate vaccines were introduced into the UK national immunisation schedule in November 1999. Laboratory-confirmed group C cases fell from 954 in 1998/99 to 64 in 2003/04 (34% to 4% of total IMD cases, respectively) (Fig. 1-2) (Gray et al., 2006). Such programmes were also adopted by several other European countries and, by 2004, notable decreases in the proportion of group C disease were observed throughout Europe. The UK incidence of group C disease has remained low (<1/100,000) in the years since (Fig. 1-2) (Trotter et al., 2007b; Borrow et al., 2013).

In 2013, 2014 and 2015, sequential outbreaks of group C disease were observed throughout northern Nigeria (Funk et al., 2016; Chow et al., 2016). In 2015 alone, >6000 suspected cases were recorded. Cultured isolates yielded a finetype of C:P1.21-15,16:F1-7:ST-10217. This subtype is uncommon and the ST is not assigned to a CC. Reactive vaccination with quadrivalent ACWY polysaccharide vaccines was implemented in 2015, however, the impact of this intervention is not yet fully determined (Chow et al., 2016). A subsequent genomic study of group C epidemic isolates in neighbouring Niger yielded the same ST (Kretz et al., 2016).

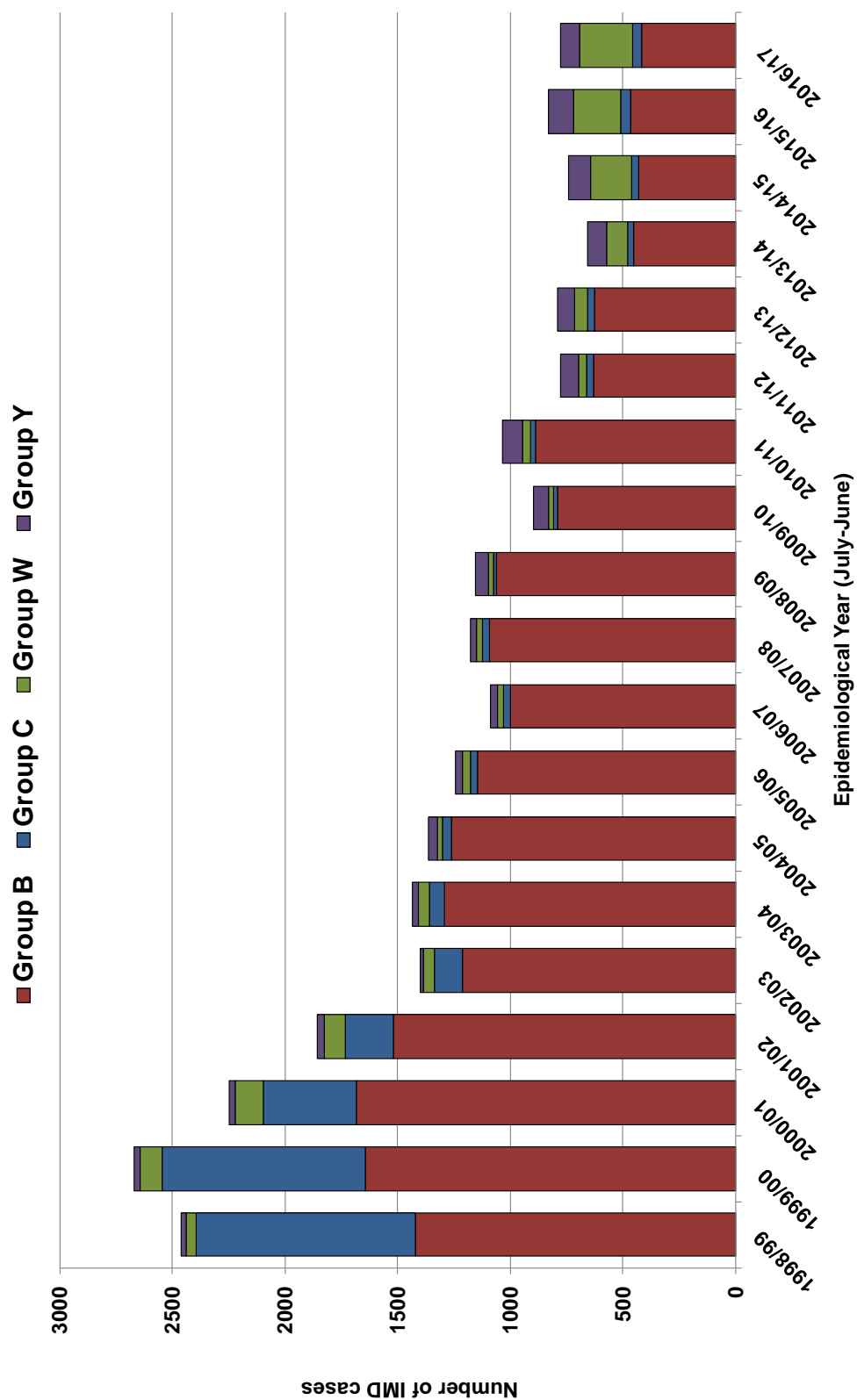


Figure 1-2: Number of IMD cases confirmed by PHE MRU from 1998/99 to 2016/17 by capsular group. Group B has been the most common group in each year since 1998/99. A notable reduction in group C cases was observed following introduction of the group C conjugate vaccine in 1999/2000. Since 2012, a significant increase in group W disease has occurred primarily due to the expansion of a hyper-virulent ST-11 complex sub-lineage.

1.7.3 Group W

Throughout the twentieth century, group W disease was reported at a low level globally in relation to the other hyper-invasive capsular groups (Silva et al., 2012). Prior to 2000, group W disease represented $\leq 10\%$ of reported IMD cases in the US, South America, Europe and Africa (Nicolas et al., 2005; Safadi et al., 2013; Baccarini et al., 2013; Gray et al., 2006).

In 2000, however, an outbreak of group W disease occurred amongst Hajj pilgrims in Saudi Arabia. The cramped conditions associated with the Hajj and the subsequent return of pilgrims to their home countries facilitated the global spread of the causative clone (W:2a:P1.5,2:ST-11). Localised outbreaks of group W disease occurred throughout Europe (primarily in the UK and France), the Middle East, Africa and East Asia (Aguilera et al., 2002; Lingappa et al., 2003; Wilder-Smith et al., 2003; Ceyhan et al., 2012). In 2002, Burkino Faso suffered a severe epidemic of over 12,000 cases (Koumare et al., 2007). These group W outbreaks featured characteristically high case fatality rates (e.g. 20% UK, 37% Singapore) and the age distribution tended towards children and young adults (Wilder-Smith et al., 2003; von Gottberg et al., 2008). Vaccination recommendations for Hajj pilgrims were changed from bivalent (groups A/C) to quadrivalent (groups A/C/W/Y) vaccines in 2002 (Karima et al., 2003). In subsequent years, this 'Hajj strain' (W:2a:P1.2,5:ST-11) became an established endemic strain throughout Africa.

Since 2010, increases in endemic group W disease caused by CC11 have also been observed in Latin America (particularly Chile and Argentina) and Western Europe (Fig 1-2) (Abad et al., 2014; Ladhani et al., 2015a; von Gottberg et al., 2008). These increases have prompted the introduction of quadrivalent conjugate vaccines in Chile, Argentina and the UK (Campbell et al., 2017; Borrow et al., 2017). In 2015, a detailed whole genome comparative analysis of a global panel of CC11 group W strains was performed. The results revealed clear distinctions between so called "Hajj strain" that caused disease in the early 2000s and the more recent strains causing high disease rates in South America and the UK (Lucidarme et al., 2015). The recent UK strains were shown to have evolved from the South American clones and expanded within the UK.

Subsequently, high-resolution MLST analysis (n=1546 loci) of UK and Swedish isolates from children returning from an international scout convention in Japan identified a distinct CC11 strain (dubbed the '2013 strain') that had expanded from the recent UK and South American epidemic strains and continues to cause increasing numbers of disease cases in the UK as well as other European countries and Australia (Lucidarme et al., 2016; Martin et al., 2016; Knol et al., 2017). Cases caused by the recent CC11 strains were observed across a wide range of age groups, however, a significant burden was seen in those ≥ 45 years of age (Ladhani et al., 2015a; Martin et al., 2016)

1.7.4 Group Y

In relation to strains of other capsular groups, group Y strains cause disease in a geographically limited area, being generally prominent in North America and Europe only. In North America, incidence of group Y disease was historically low until the 1990's when increases in disease rates saw group Y account for approximately a quarter of IMD in the United States (Rosenstein et al., 1996).

Group Y strains cause ~5% of IMD in Europe on average, however, a 10% increase in mean annual increase in notification rate was observed between 2000 and 2014 (Whittaker et al., 2017) with notable recent increases in Scandinavia, Italy and the UK (Broker et al., 2014). In 2009/10 in the UK, a corresponding increase in group Y carriage was observed in university students (Bidmos et al., 2011). Recent European strains have been relatively homogenous, with the majority belonging to CC23, however, core genome MLST analyses have revealed several sub-clones that differ in predominance between the UK and Sweden (Törös et al., 2015). Group Y strains exhibit a distinctive pathogenicity with a relatively large proportion of disease cases featuring pneumonia and/or other less common symptomologies (Ladhani et al., 2012a; Säll et al., 2017). This may in part be due to the skewed age distribution towards those ≥ 65 years of age, in which co-morbidities and co-infections may impact the nature and likelihood of infection. Unlike group W, however, few group Y cases are observed in infants and children and the indirect herd protection effect provided by immunising adolescents (the primary carriers) with quadrivalent polysaccharide conjugates may not be as evident against older age groups.

1.7.5 Group B

Since the 1960's and 70's, group B has been the predominant invasive group across many countries of Europe, the Americas and Australasia (Trotter et al., 2007b; Harrison et al., 2009; Baccarini et al., 2013). In recent years, particularly high mean annual group B disease incidences (1.4 – 2.36/100,000) have been seen in the UK, Ireland, the Netherlands and New Zealand, however, this the incidence has been gradually reducing year on year in the UK and other countries (Sridhar et al., 2015; Ladhani et al., 2012b).

Invasive group B strains are genetically more diverse than those of other capsular groups with a number of globally-dominant clonal lineages. Strains of the ST-32 complex lineage have been responsible for a significant proportion of European group B endemic disease over the past four decades (Bygraves et al., 1999; Brehony et al., 2014; Trotter et al., 2007b). In addition, a number of hyper-invasive ST-32 complex strains, belonging to several different STs, have caused prolonged outbreaks in Norway, UK, US (B:15:P1.7,16), Cuba, South America (B:4:P1.19,15 and B:15:P1.7,3) and France (B:14:P1.7,16) (Caugant et al., 1986; Diermayer et al., 1999; Rouaud et al., 2006). ST-41/44 complex strains represent a large proportion of the global group B disease burden (European Centre for Disease Prevention and Control, 2012). A high group B incidence reported in New Zealand was due to a prolonged outbreak of a ST-41/44 complex strain (B:P1.7-2,4:ST-42), which began in 2000 (Dyet and Martin, 2005). This led to the development and implementation of an OMV vaccine (MenNZB) against the causative strain in 2004 (Sridhar et al., 2015). ST-269 complex strains cause a large proportion of endemic disease, especially in the UK, as well as a rapid expansion in Quebec, Canada leading to vaccination of one region in 2014 (Lucidarme et al., 2009; Law et al., 2006; De Wals et al., 2017).

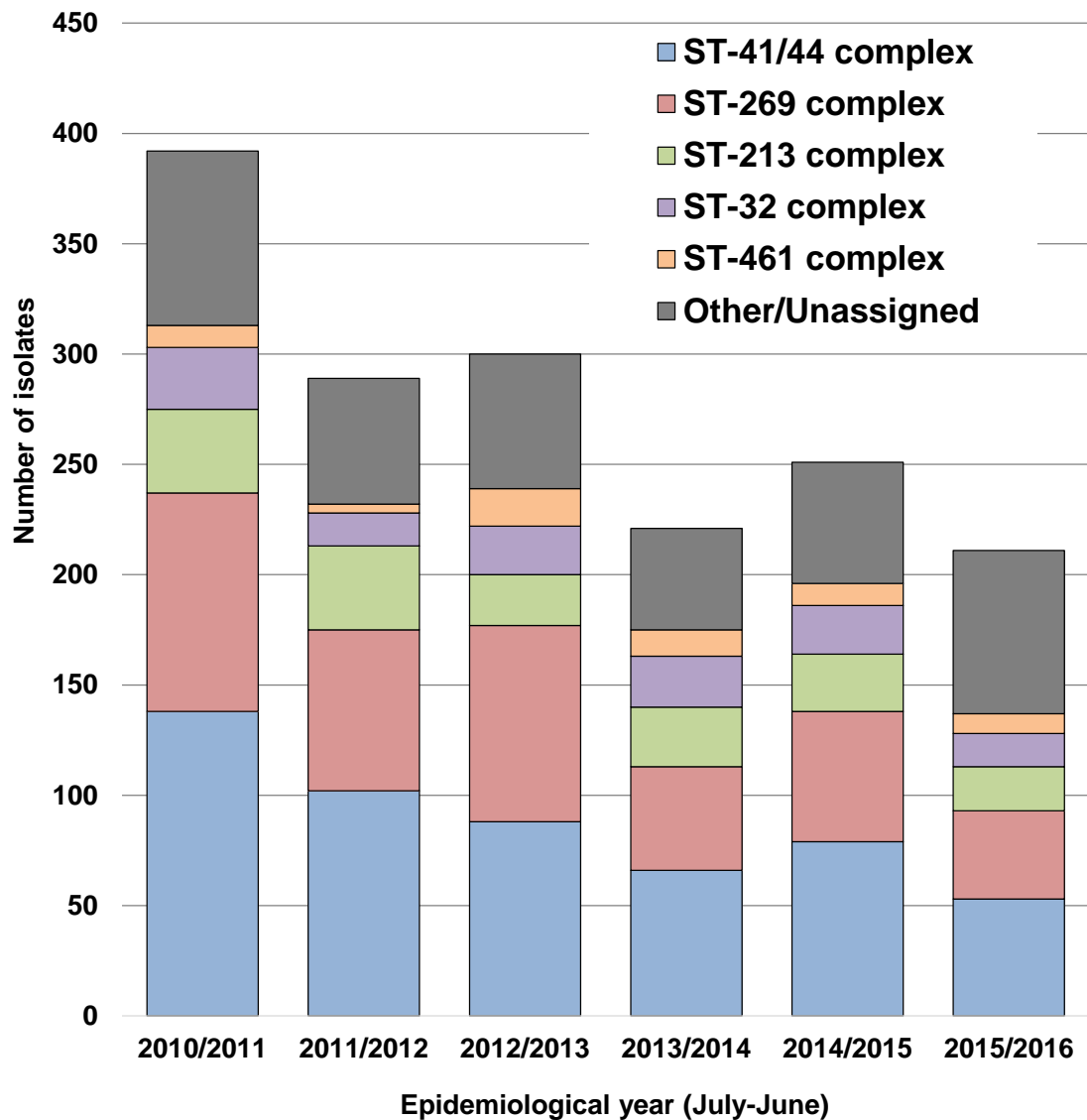


Figure 1-3: Distribution of predominant hyper-invasive CCs amongst group B isolates in E&W between 2010 and 2016. Data sourced from Meningitis Research Foundation’s Meningococcus Genome Library (MRF MGL, <https://www.meningitis.org/research-projects/mrf-meningococcus-genome-library>). “Unassigned” isolates are those which an ST could not be assigned due to at least one incomplete/interrupted MLST locus in the genome assembly.

In E&W, between July 2010 and June 2016, 63.6% of invasive group B isolates with defined STs belonged to one of the three aforementioned hyper-invasive lineages (Fig. 1-3). The ST-213 complex was the third most prevalent lineage representing a further 10.3% of characterised group B culture cases during this period.

In E&W, group B disease is predominant in all ages, however, the majority of group B cases are seen in those under 10 years and those between 15-19 years of age. (Hill et al., 2015; European Centre for Disease Prevention and Control, 2012; Ladhani et al., 2012b). Interestingly, variation can be seen in the age distribution of different common group B lineages with, for example, ST-32 complex disease more common in older children (5-14 years) (Brehony et al., 2014).

Fatality rates for group B disease are around 5-10% in industrialised settings, however, particularly transmissible and virulent strains (i.e. during an outbreak) can cause substantially higher case fatality rates (Rouaud et al., 2006; Sridhar et al., 2015).

1.8 Meningococcal Vaccines

1.8.1 Capsular polysaccharide antigens and T-cell independent immunity

The late 1960s saw the development of improved purification methods to extract high-molecular weight group A, B and C polysaccharides. Following testing in animal models, the group A and C polysaccharides were shown to generate robust group-specific bactericidal antibodies in human adult volunteers (Gotschlich et al., 1969; Artenstein et al., 1970a, 1971; Gotschlich et al., 1972). Subsequent vaccination programmes among US army recruits resulted in substantial reductions in group C meningococcal disease among the vaccinated camps (Artenstein et al., 1970b; Gold and Artenstein, 1971). Similar field trials evaluating group A polysaccharide vaccines in Egypt, Finland and the Sudan reported high immunogenicity and group-specific reductions in disease (Erwa et al., 1973; Wahdan et al., 1977; Makela et al., 1975).

Despite promising results from these initial studies, a number of substantial limitations were subsequently revealed. Perhaps most critically, it was determined that the

immunogenicity of these vaccines was age-specific. Group C polysaccharide vaccines do not elicit sufficient immunity to protect children under 2 years of age (Taunay et al., 1974). Furthermore, group C polysaccharide elicits hypo-responsiveness, reducing the immunogenicity of subsequent doses and precluding their use in routine immunisation programmes (Gold et al., 1979; Poolman and Borrow, 2011). Evidence suggests this may be due to the immaturity of the splenic marginal zones and/or apoptosis of memory B lymphocytes following immunisation in infants (Brynjolfsson et al., 2012).

Following immunisation with group A polysaccharide, infants under 12 months produce ten-fold lower levels of antibody than adults; less than putative protective levels (2µg/mL) (Wilkins and Wehrle, 1979; Gotschlich et al., 1972). Short-term protection is seen in infants over 3 months of age, however, vaccine efficacy can reduce markedly within 2-3 years when given to those under 4 years (Reingold et al., 1985). Even following booster doses, antibody titres in infants aged <12 months wane within a year, however, older children and adults respond well to boosting making it suitable for routine use in these populations (Lepow et al., 1977; Kayhty et al., 1980).

Meningococcal capsular polysaccharides, like most bacterial carbohydrates, are known as T-cell independent (TI) antigens (specifically TI-2 antigens) as they do not bind to MHC class II proteins and are not, therefore, presented on the surface of antigen presenting cells (APC, e.g. dendritic cells, macrophages) for T-cell recognition and activation (reviewed by Avci et al. 2011). TI-2 antigens activate B-cells directly through cross-linking of the B cell receptors (BCR) resulting in a largely humoral immune response characterized by IgM antibody generation and limited generation of memory B cells, hindering the development of immune memory (Beuvery et al., 1982).

Despite these limitations, several capsular polysaccharide vaccines containing A, C, W and Y polysaccharides have been licensed. Polysaccharide vaccines have been successful in maintaining low IMD incidence within specific at-risk populations, such as army recruits, and as part of reactive vaccine campaigns in Africa (Brundage et al., 2002; Artal, 2015). Crucially, however, the development and licensure of group B polysaccharides has been hampered by the structural similarity between the group B polysaccharide and human neuronal cells (Finne et al., 1983). This similarity reduces its

ability to elicit bactericidal antibodies and raises concerns about possible auto-immune reactions following vaccination.

1.8.2 Polysaccharide conjugates and T-cell dependent immunity

Many of the disadvantages inherent in the use of polysaccharide antigens were overcome through the development of protein-conjugated *Haemophilus influenzae* type b polysaccharide vaccines. These conjugated vaccines elicit stronger immune responses characterised by high avidity antibodies as well as the induction immune memory (Kelly et al., 2004; Pichichero, 2013). The conjugation of bacterial polysaccharides to immunogenic carrier proteins promotes the uptake, processing and MHC-II-mediated presentation of the polysaccharide by APCs. Subsequent MHC-II recognition by carbohydrate-specific CD-4 T cells triggers cytokine release and the proliferation and differentiation of B cells. The maturation of the B cells involves antibody isotype switching (e.g. from IgM to IgG), production of higher avidity antibodies and increases in the number of memory B cells, which are necessary for immune memory responses upon repeated antigenic exposure (Kelly et al., 2006).

The first meningococcal conjugate vaccines were developed and introduced in the UK in the late 1990s. Three group C polysaccharide conjugates (two conjugated to modified diphtheria toxoid CRM₁₉₇ and one to tetanus toxoid (TT)) were introduced in response to a steep increase in group C disease in the preceding years (Miller et al., 2001). Two of the three vaccines (NeisVac-C™, TT-conjugated and Meningitec™, CRM₁₉₇-conjugated) had been licenced based on immunogenicity data derived from rSBA analysis of post-immune sera using a putative protective titre of ≥ 8 (Borrow et al., 2001). The SBA data for the other vaccine (Menjugate®, CRM197-conjugated) was mostly generated using hSBA (MacLennan et al., 2000).

Acceptable safety profiles, induction of immunological memory and high levels of protective immunity in infants and toddlers were demonstrated after one or two doses of either vaccine (Richmond et al., 1999; Bramley et al., 2001; Richmond et al., 2001; MacLennan et al., 2000). The hypo-responsiveness previously observed with repeated polysaccharide vaccination was not seen with the conjugate vaccines and prior polysaccharide vaccination did not impair the booster response of conjugated vaccines

(MacDonald et al., 1998; Poolman and Borrow, 2011). Additionally, plain polysaccharide vaccines were able to boost the response primed by the conjugated vaccines (Richmond et al., 2001).

In late 1999, the first group C conjugate vaccine was added to the national infant programme at a 2-, 3- and 4-month schedule. This was rolled out alongside a catch-up campaign for those aged up to 18 years (Miller et al., 2001). The group C conjugate vaccines had a dramatic effect on group C disease in the UK (see section 1.7.2). In 2006, the UK infant schedule was changed to a two dose primary (3 and 4 months) and a booster at 12 months of age in order to extend direct protection into the second year of life (Borrow et al., 2010). This was reduced further in 2013 to a single priming dose and 12 month booster but with the introduction of an adolescent booster (13-14 years and university entrants) in order to provide protection to those who had only been vaccinated during infancy (i.e. those not part of the initial catch up campaign) and to provide herd protection (Findlow and Borrow, 2015). Finally, in 2016, the single primary group C dose was removed from UK routine schedule. It was deemed unnecessary due to the low numbers of group C cases and continuing herd protection provided by adolescent vaccination.

Since the success of group C conjugate vaccines, multivalent glyco-conjugates (ACWY and CY) have been licenced in many countries and possess TT or diphtheria derived carrier proteins. The usage depends on the licence restrictions but many are used in infants e.g. Menveo™ (ACWY-CRM197) licenced from 2 months in US and Nimenrix™ (ACWY-TT) is licenced from 6 weeks of age in EU (Ali et al., 2014).

In the UK in 2015, the adolescent MenC conjugate dose was changed to an ACWY conjugate in response to the increase in group W and Y disease in the UK (Campbell et al., 2017). Clinical studies in teenagers who had been primed with a conjugate MenC vaccine in infancy found a very high response rate (>98%) one month post-boosting. Those primed and boosted with TT-conjugated vaccines exhibited the highest group C SBA titres (Ishola et al., 2015).

The TT-conjugated MenAfriVac has all but eliminated group A disease from vaccinated areas in sub-Saharan Africa and also elicited anti-tetanus serological responses leading

to a significant reduction in neonatal tetanus (Borrow et al., 2015; Diomande et al., 2015). Despite this success, the persistence of non-group A disease throughout sub-Saharan Africa has prompted research into the development of affordable mono-valent and multi-valent vaccines for targeting strains of other capsular groups in resource-poor settings (Micoli et al., 2013).

1.8.3 Outer Membrane Vesicles Vaccines

N. meningitidis has the ability to bleb and release OMVs during proliferation. OMVs contain a medley of outer membrane and periplasmic structures, many of which are immunogenic protein antigens (Vaughan et al., 2006). In the 1970s, the lack of polysaccharide vaccines against group B strains prompted research into the use of OMVs as vaccines (Holst et al., 2013). These early vaccines were derived from epidemic strains using deoxycholate detergent membrane extraction, which reduces the LOS content, therefore attenuating their toxicity (Acevedo et al., 2014; Holst et al., 2013). These vaccines exhibit good safety profiles and generate strong SBA responses against the strains from which they are derived and those expressing the same PorA VR2 epitope. However, against strains which express heterologous PorA variants, monovalent OMV vaccines elicit limited cross-protective immunity, especially in infants (Martin et al., 2006; Thornton et al., 2006; Nokleby et al., 2007; Holst et al., 2009).

Three monovalent OMV vaccines are currently licenced for the prevention of meningococcal disease. VA-MENGOC-BC® was developed by the Finlay Institute in Cuba and its OMV is derived from the CU385 epidemic strain from central America (B:4:P1.19,15:ST-32 complex). The vaccine was introduced into the Cuban vaccine schedule in 1989, initially for those up to six years of age with a reported efficacy of 92.56% (Sierra et al., 1991). A trial of VA-MENGOCOC-BC® in Brazil, however, reported lower efficacy (-37 to 74%) in a setting in which only 58.4% of invasive strains possessed the matched the VR2 subtype (de Moraes et al., 1992).

The Norwegian-produced MenBvac® was developed from the hyper-invasive, European strain H44/76 (B:15:P1.7,16:ST-32 complex) (Rosenqvist et al., 1995). Trial results have demonstrated high vaccine efficacy against the epidemic strain (Holst et al., 2013). The efficacy correlated well with immunogenicity data in adolescents which showed a

significant increase in geometric mean titres following two doses (2.4 to 19.0). Titres had, however, waned to pre-vaccination levels after 12 months (Holst et al., 2003).

Lastly, an international collaboration between the Norwegian Institute of Public Health, the New Zealand government and Chiron Vaccines resulted in the licensure of MeNZB™ to tackle an epidemic of the New Zealand NZ98/254 strain (B:4:P1.7-2,4:ST-41/44 complex) (Thornton et al., 2006).

Whilst these vaccines have been successful in controlling single-strain epidemics, the immunodominance of the PorA antigen precludes the use of these vaccines against diverse endemic strains. Despite these limitations, OMVs have proved themselves to be an effective platform for antigen delivery and new generations of OMV vaccines are being developed in an attempt to improve immunogenicity and strain coverage. Many recent formulations contain native OMVs which are naturally-blebbed and passively collected from the growth medium. The use of naturally conformational OMVs overcomes many shortcomings of detergent OMV extraction such as the loss of vesicle integrity, increased aggregation and the removal of potentially immunogenic phospholipids and lipoproteins (van de Waterbeemd et al., 2010).

In order to overcome the limited cross-protection, genetic engineering has been used to expand the PorA repertoire of recent OMV vaccines (Van der Ley and Poolman, 1992). HexaMen and NonaMen, for example, consist of two or three deoxycholate-extracted OMVs each expressing three different PorA variants, respectively. The inclusion of multiple PorA variants increases the proportion of strains covered (Vermont et al., 2003; Kaaijk et al., 2013).

Several other candidate OMVs have tailored antigenic makeup and/or increased expression of constitutive antigens (Koeberling et al., 2009; Weynants et al., 2009; Pajon et al., 2013; Norheim et al., 2015). Additionally, a number of vaccine formulations, including the licenced group B vaccine Bexsero® (4CMenB), contain OMVs alongside recombinant proteins and/or glyco-conjugated polysaccharides in order to increase strain coverage and immunogenicity (Gorringe and Pajon, 2012; Tunheim et al., 2014; Block et al., 2015).

1.8.4 Recombinant Protein Vaccine Antigens

1.8.4.1 Identification of protein antigen candidates

The limited scope of protection offered by first generation, monovalent OMV vaccines prompted researchers to identify individual sub-capsular protein antigens which are relatively conserved between invasive strains and/or exhibit substantial cross-reactive immunogenicity. Developments in genomic technologies and the sequencing of the first two complete meningococcal genomes in 2000 provided researchers unprecedented insight into the workings of the meningococcal cell (Tettelin et al., 2000; Parkhill et al., 2000). With this came the opportunity to use the genomic data as a starting point in identifying promising surface-exposed vaccine candidates. Using a technique known as Reverse Vaccinology, researchers at Chiron Vaccines (subsequently Novartis and GlaxoSmithKline) based in Siena, Italy carried out comprehensive *in silico* analyses of the genome of group B strain MC58 in order to identify genes with features and motifs indicating cell surface localisation such as the LXXC lipobox motif. The group expressed 350 such proteins in *Escherichia coli*, which were then used to immunise mice to assess immunogenicity (Pizza et al., 2000). These proteomic and genotypic analyses of surface proteins have yielded a number of promising vaccine antigen candidates.

1.8.4.2 Factor H-Binding Protein (GNA1870 / LP2086)

Factor H-binding protein (fHbp) was among the prospective antigens identified by the Siena group through the use of Reverse Vaccinology (Masignani et al., 2003). Concomitantly, this 28kDa protein was identified independently by a New York-based research group at Wyeth Vaccines (now Pfizer Vaccines) using detergent extraction and ion chromatography and was given the designation Lipoprotein 2086 (LP2086) (Bernfield et al., 2002; Fletcher et al., 2004).

NMR spectroscopy and X-ray crystallography revealed fHbp's dual domain structure, which consists of an N-terminal 'taco shaped' β - sheet (residues 1-137) and a C-terminal anti-parallel β - barrel (residues 138-255, Figure 1-4). The wild type protein is lipidated at the N-terminal and is anchored to the outer membrane via an

serine/glycine linker of varying length (approximately 20 residue) allowing the antigen to sit away from the membrane (Figure 1-4) (Mascioni et al., 2009, 2010; Cantini et al., 2009; Cendron et al., 2011). This is likely to reduce any masking of immunological epitopes by polysaccharide capsule and other membrane components, however, there is evidence to suggest that the LOS layer may prevent the binding of monoclonal antibodies to epitopes adjacent to the outer membrane (Mascioni et al., 2009, 2010).

Considerable sequence variation exists between the different fHbp peptides harboured by invasive and non-invasive strains. Individual variants fall into three broad groups based on amino acid sequence similarity: variant groups 1, 2 and 3. Variants within each group exhibit >81% amino acid identity whilst similarity between variant groups 1 and 3 is as low as 63% (Brehony et al., 2009; Maignani et al., 2003). In an alternate naming scheme, variant groups 2 and 3 are collectively referred to as 'subfamily A', reflecting their relative similarity, whilst variant group 1 is named 'subfamily B' (Fletcher et al., 2004).

Epitope mapping studies have shown that most subfamily/variant group specific residues are located on the extracellular face of the protein, which is likely to be evidence of immunological selection pressures (Cantini et al., 2009; Mascioni et al., 2010). The identification and analysis of additional fHbp peptide variants, in particular natural chimeras, revealed a distinctly modular structure in which small invariant peptide regions separate five large variable segments (Beernink and Granoff, 2009; Pajon et al., 2010). Each segment (A to E) of a specific peptide variant, is given either an α or a β designation (or 1 or 2, respectively) (Beernink and Granoff, 2009). Using the unique combinations of the five segments, Beernink and Granoff (2009) divided individual fHbp peptides into six modular groups (I to VI), later expanded to nine groups (I-IX) (Beernink et al., 2009; Pajon et al., 2010).

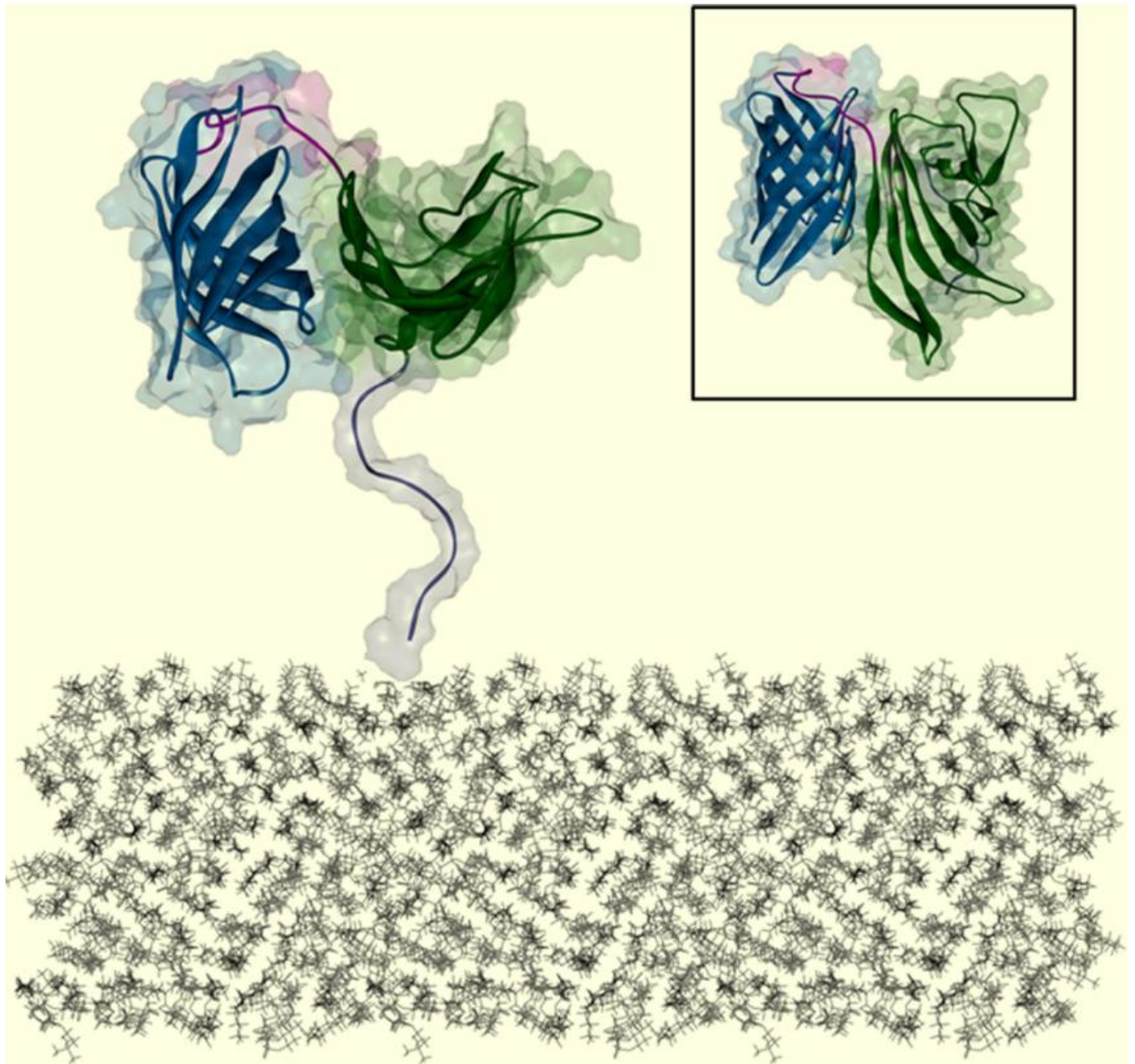


Figure 1-4: A schematic representation of the fHbp peptide (variant 1.55) as expressed from the surface of the meningococcal outer membrane.

White: Serine/Glycine 'tether' anchoring the protein to the outer membrane. Green: N-terminal β -sheet. Blue: C-terminal β -barrel. Pink/red: linker between the two main domains. Boxed inset image: representation of the peptide surface facing away from outer membrane.

Image reproduced from McNeil, L. K., Zagursky, R. J., Lin, S. L., Murphy, E., Zlotnick, G. W., Hoiseth, S. K., Jansen, K. U. and Anderson, A. S. (2013). 'Role of Factor H Binding Protein in *Neisseria meningitidis* Virulence and Its Potential as a Vaccine Candidate To Broadly Protect against Meningococcal Disease'. *Microbiology and Molecular Biology Reviews*, 77 (2), pp.234–252., with permission from the American Society for Microbiology.

Most of the differences between subfamily A and B are seen in the C-terminal domain, whilst the N-terminal domain is important in distinguishing the two groups of subfamily A (Brehony et al., 2009; Murphy et al., 2009). The N and C domains are subject to interchanging recombination resulting in variants with combinations of domain types with unique signature residues. In a 2009 study, four distinct subfamily A N/C-terminal combinations were identified, namely N1C1, N1C2 (variant group 3), N2C1 and N2C2 (variant group 2) (Murphy et al., 2009). Subfamily B variants were divided into three further N-terminal domain types: N4, N5 and N6 (Murphy et al., 2009).

The primary function of fHbp was first reported by Madico *et al* in 2006. Knock out strains and western blotting show fHbp to be a ligand for human fH, a negative regulator of the alternative complement pathway (Madico et al., 2006). Recruitment of fH to the cell surface by fHbp increases the survival of meningococci in human blood and serum (Madico et al., 2006; Welsch et al., 2008; Seib et al., 2009). The protein binds to domain 6 and partially at domain 7 of human fH (Schneider et al., 2009). Peptides of all variant groups can bind fH and recruitment of fH correlates with the level of fHbp at the bacterial surface (Seib et al., 2011; Madico et al., 2006). The fH affinity of different fHbp variants can, however, vary independently of expression level (Seib et al., 2011). Studies involving diverse isolate panels have observed lower expression of subfamily A variants in relation to subfamily B peptides (Hong et al., 2012; Biagini et al., 2016). In 2008, a study suggested that strains with higher levels of fHbp expression may exhibit greater dependence on the lipoprotein for immune resistance than those with low fHbp expression (Welsch et al., 2008). These differences in fH affinity and surface expression of fHbp may be associated with the differential expression of alternate fH ligands among meningococcal strains such as Neisserial Surface Protein A, PorB3 and sialylated LOS (Lewis et al., 2012).

To date, almost all characterised invasive isolates, regardless of capsular group, possess fHbp. The level of surface expression can, however, vary considerably and, in several studies, strains were divided into high and low expressors (Beernink et al., 2007; Seib et al., 2010; Maignani et al., 2003; Fletcher et al., 2004). Expression level can influence the level of bactericidal activity with high expressors being more susceptible to

bactericidal killing (Jiang et al., 2010; Pajon et al., 2010). A small proportion of invasive meningococcal strains harbour *fHbp* alleles yielding truncated peptides and an even smaller number (typically <0.2%) lack an *fHbp* locus (Lucidarme et al., 2011a). In these strains, the locus is replaced by a gene commonly observed in *N. lactamica* (NLA18150) encoding a putative opacity protein (Lucidarme et al., 2013a).

Vaccination with fHbp generates a variant group-specific response, i.e. antibodies elicited by variant group 2 fHbp peptides are generally not bactericidal against strains expressing variant group 1 variants (Pajon et al., 2010; Maignani et al., 2003; Fletcher et al., 2004). In one study, this principle was tested using transgenic mutants possessing different subfamily B variants expressed from a constitutive promoter (Brunelli et al., 2011). Pooled anti-fHbp variant 1.1 serum from adults exhibited strong SBA reactions against all mutants expressing variant 1 peptides, however titres were relatively lower against the heterologous variants (i.e. not 1.1). Pooled sera from infants immunised with recombinant fHbp variant 1.1 were poorly cross-reactive against strains with other variant 1 peptides after three doses (2, 4 and 6 months) and showed limited cross-reactivity after a booster dose (12 months) (Brunelli et al., 2011). These results indicate that the cross-reactivity of fHbp within variant groups could be less prominent in infants due to the under-developed immune system and suggests that fHbp alone may not provide as broad strain coverage in this age group as in adults. There is evidence that expression level could impact on the cross-reactivity of antibodies raised against heterologous variant groups, with higher expressers more susceptible to antibodies raised against fHbp of a different variant group (Pajon et al., 2010).

Several variant-specific monoclonal antibodies have been described and the corresponding epitopes contributing to bactericidal activity have been identified (Malito et al., 2013; Welsch et al., 2008; Mascioni et al., 2010). Many of these antibodies exhibit a synergistic bactericidal effect, in which binding of antibodies to epitopes at a distance of 16 to 20 Å is required to induce C1q engagement and initiation of complement-mediated bactericidal activity (Beernink et al., 2008). In 2016, Biagini *et al.* used western blotting and flow cytometry on mutant MC58 strains and determined that bactericidal antibody activity required an fHbp surface density of 757

molecules per cell. This was deemed consistent with the distance between molecules required for C1q engagement (Biagini et al., 2016).

In most strains, transcription of fHbp is driven by a promoter sequence upstream of *fHbp*. Under oxygen-limiting conditions, transcription of *fHbp* is upregulated in a fumarate and nitrate reductase-dependent manner (Oriente et al. 2010). Conversely, *fHbp* is upregulated in iron replete conditions (Sanders et al., 2012). The gene can also be transcribed from the promoter of the upstream *cbba* gene as a bi-cistronic transcript, however, this has only been observed in strains of the ST-32 complex (Oriente et al., 2010; Sanders et al., 2012). The *fHbp* promoter also features a RNA thermosensor which, at low temperatures, can restrict translation of the peptide (Loh et al., 2013).

Differing nomenclatures are currently used to describe allelic variation in the *fHbp* gene and the corresponding peptides. The Sienna group used arbitrary numbers to identify unique nucleotide and peptide variants as they were discovered. These IDs are preceded by the variant group to which the variant belongs separated with a full stop (e.g. '1.15' refers to variant 15 within variant group 1) (Bambini et al., 2009). In this system, the original variant identified through analysis of MC58 is 1.1. The New York group (currently Pfizer Vaccine Research) established a similar but unrelated system in which arbitrary, unique numeric identifiers are immediately preceded by the subfamily to which the variant belongs (e.g. B44, refers to variant 44 within subfamily B) (Fletcher et al., 2004). Following the incorporation of fHbp into University of Oxford's Bacterial Isolate Genome Sequence Database (BIGSdb) typing repertoire, the identifiers assigned by Sienna were adopted for the most common variants that were initially discovered. Additional nucleotide and peptide variants that were subsequently submitted to the database were given successive numbers and these are then cross referenced with the alpha numeric 'Pfizer system' (Brehony et al., 2009).

1.8.4.3 *Neisseria* Adhesin A

The use of reverse vaccinology also led to the identification of *Neisseria* Adhesin A (NadA, originally named GNA1994), an oligomeric protein which is strongly immunogenic in rats and humans and has been shown to play an integral role in

adhesion to and invasion of host epithelial cells (Comanducci et al., 2002; Capecchi et al., 2005; Findlow et al., 2010).

NadA exhibits significant amino acid sequence variation and allelic variants can be clustered into three broad groups (Bambini et al., 2014). Group I contains two subgroups NadA-1 and NadA-2/3 which share >90% sequence homology and are immunologically cross-reactive (Comanducci et al., 2002). Group I alleles are commonly found among a limited number of albeit predominant invasive lineages, namely the ST-32 complex, ST-11 complex and ST-8 complex. Group II contains subgroups NadA-4/5 and NadA-6 which are mostly restricted to carrier strains or the cc213 invasive lineage. As such, NadA is not as widely represented among invasive strains as fHbp (Comanducci et al., 2004; Bambini et al., 2009).

Expression of NadA can differ 100 fold between harbouring invasive strains and expression is influenced by phase variation mediated by homopolymeric tracts in the gene promoter region (Beernink et al., 2007). NadA expression is naturally repressed by the NadR regulator. During colonisation of the nasopharynx, NadA expression is de-repressed in the presence of 4-hydroxyphenyl acetate, a phenol ester commonly found in human saliva, by inhibiting the DNA-binding capability of NadR. This finding raises questions about the validity of SBA results generated *in vitro* in the absence of 4-hydroxyphenyl acetate (Metruccio et al., 2009).

1.8.4.4 Neisserial Heparin Binding Antigen

Neisserial Heparin Binding Antigen (NHBA, originally named GNA2132) is a surface-located protein that was also discovered by reverse vaccinology through the identification of an associated LXXC motif and was initially found to be capable of eliciting bactericidal antibodies in mice (Pizza et al., 2000). A subsequent immunogenicity study using anti-NHBA murine sera generated conflicting results (Welsch et al., 2003). Despite demonstrable antibody binding, complement deposition and rSBA bactericidal activity, the anti-NHBA antiserum only killed 2 out of 7 strains when tested with human complement. Interestingly, the strain from which the recombinant NHBA variant was derived was one of the five hSBA-resistant strains. The anti-NHBA serum did, however, provide passive protection in intraperitoneally-infected

rats, suggesting OPA may play a protective role (Welsch et al., 2003). Serruto *et al.* used dot blotting and hSBA to demonstrate NHBA-specific killing by human convalescent sera (Serruto et al., 2010). Using affinity chromatography, the protein was shown to bind Heparin via a conserved, central Arg-rich region, which increases serum resistance in the SBA. Furthermore, NHBA was shown to be cleaved from the surface by the NalP protease (in some strains) and human Lactoferrin at sites adjacent to the Arg-rich region (Serruto et al., 2010). Recently, Partridge *et al* demonstrated a significant NHBA contribution to SBA elicited by 4CMenB, especially in conjunction with anti-fHbp antibodies (Partridge et al., 2017). The NHBA-encoding gene is found in the all invasive strains, however, a small proportion of strains harbour NHBA variants with frameshifts and/or insertion sequences which disrupt translation (Jacobsson et al., 2006; Lucidarme et al., 2010; Bambini et al., 2009).

1.8.5 Recombinant Antigen Vaccines

1.8.5.1 4CMenB

4CMenB is a protein vaccine comprising three recombinant proteins (50 µg of each) and an OMV from the New Zealand outbreak group B strain NZ98/254 (25 µg) adjuvanted with aluminium hydroxide. Five recombinant proteins were identified among the MC58 genome using reverse vaccinology (Pizza et al. 2000). fHbp (GNA1870), NHBA (GNA2132), NadA (GNA1994), *Neisseria* ubiquinone binding protein (GNA1030) and a yet unnamed protein (GNA2091) were selected due to their collective presence among diverse group B strains as well as the ability of the corresponding antisera to elicit SBA activity and/or passive protection in infant mouse models (Giuliani et al., 2006; Pizza et al., 2000; Comanducci et al., 2002; Serruto et al., 2010; Masignani et al., 2003).

In order to facilitate large-scale production of these recombinant proteins, four of the antigens are presented as two fusion proteins: NHBA is fused with NUBp and fHbp is fused with GNA2091. The fusion of these proteins also appears to increase the SBA activity induced by the individual antigens (Giuliani et al., 2006). Early human trials reported greater immunogenicity with formulations containing the NZ98/254 OMV (4CMenB / rMenB + OMV) compared to the recombinant antigens alone (5CVMB /

rMenB) or those with the Norwegian 44/76 SL strain OMV (rMenB + NW OMV) (Findlow et al., 2010; Snape et al., 2010; Biagini et al., 2016).

As with other meningococcal vaccines, the efficacy of 4CMenB was assessed using immunogenicity data measuring the production of protective bactericidal antibodies in vaccinees (Bai et al., 2011). Clinical trials were designed to assess the immunogenicity of each individual antigenic component by using hSBA assays against multiple reference strains, each possessing and expressing one of the four antigens but mismatched (i.e. expresses heterologous variants) for the remaining three antigens. A recent study of a small number of immunised adults found that these reference strains exhibit higher susceptibility to immune serum than a diverse panel of recent invasive strains (Giuntini et al., 2017) suggesting the use of the reference strains may over-estimate vaccine efficacy.

The key Phase II and III trials that led to the licensure of 4CMenB are summarised in Table 1-1. Strong SBA responses have been shown to last at least six months in healthy adolescents and adults after two doses at one to six months apart (Kimura et al., 2011; Santolaya et al., 2012; Snape et al., 2013). Santolaya et al. (2013) found that adolescent titres remained high in >64% of vaccinees when tested 18-24 months after the second or third dose (Santolaya et al., 2013). Conversely, a small study in adults found that SBA titres against a number of diverse strains waned significantly after only 4-6 months (Giuntini et al., 2017). In infants, strong SBA responses are seen after two or three primary doses given one or two months apart (Gossger et al., 2012; Findlow et al., 2010). An anamnestic response was observed following boosting at 12 months, however, waning of bactericidal antibodies was seen by 60 months, even after a secondary booster at 40 months (Vesikari et al., 2013; McQuaid et al., 2015). The vaccine shows an acceptable safety profile in all age groups but increased reactogenicity relative to other routine vaccines (RV) has been widely reported and largely attributed to the OMV component (Perrett et al., 2015; Gossger et al., 2012; Santolaya et al., 2012; Vesikari et al., 2013). No interference with commonly used vaccines was indicated, however, increased reactogenicity was observed when administered with other scheduled vaccines (Vesikari et al., 2013; Gossger et al., 2012).

Table 1-1: Key Phase II/III studies assessing the safety and immunogenicity of 4CMenB.

Age Group	Study ID	Country	No of subjects	Age	Formulation	Schedule	Endpoints	Results	Reference
Infants (<1 yr)	NCT00381615	UK	147	2 months	rMenB or 4CMenB	2, 4, 6 & 12 months	Reactogenicity and safety. hSBA titres at pre-primary, pre-booster and 1 month after 2nd, 3rd & booster.	Both vaccines tolerated and immunogenic against strains with homologous or similar antigen variants. rMenB + OMV elicits greater SBA responses.	(Findlow et al. 2010)
	NCT00433914	UK	60	6-8 months	rMenB or 4CMenB	6, 8 & 12 months	Reactogenicity and safety. hSBA titres at baseline and one month after 2nd & 3rd dose.	Both vaccines tolerated and immunogenic against strains with homologous or similar antigen variants. rMenB + OMV elicits greater SBA responses.	(Snape et al. 2010)
	NCT00721396	Europe (various)	1885	2 months	4CMenB with/without routine vaccinations (RV)s	2, 4 & 6 OR 2, 3 & 4 months	hSBA response at baseline and following third dose.	Bexsero immunogenic against reference strains with homologous variants with and without RVs using either dosing schedule. Minimal interference with RV immunogenicity.	(Gossger et al. 2012)
	NCT00657709	Europe (various)	3630	2 months	4CMenB with/without RVs	2, 4, 6 & 12 months	Lot to lot consistency. Reactogenicity and immunogenicity by hSBA assay at baseline, following third dose and following booster	Bexsero immunogenic against strains with homologous or similar antigen variants with and without RVs. Minimal interference with RV immunogenicity. Bexsero more reactogenic with RVs.	(Vesikari et al. 2013)
Toddlers (1-3 yrs)	NCT00847145	Europe (various)	2249	12 months	4CMenB with/without RVs	Booster at 12 months	hSBA responses one month after booster dose.	95-100% with hSBA titre of ≥ 5 after 12 month booster with or without RV.	(Vesikari et al. 2013)
	NCT01026974	UK	60	40-62 months	rMenB or 4CMenB	6, 8, 12 and 40 months	hSBA titres before 40 month booster and one month after booster	4CMenB titres wained up to 40 months. 4CMenB immunogenic following booster at 40 months.	(Snape et al. 2013)
	NCT01027351	UK	132	40-60 months	rMenB or 4CMenB	2, 4, 6, 12 & 40 months OR 12, 40 & 42 months OR 40 & 42 months OR 60 & 62 months	hSBA response at 60 or 63 months following various schedules and formulations.	4CMenB titres wained between booster doses at 40/42 months and 60 months. 4CMenB immunogenic following two primary doses at 60 and 62 months.	(McQuaid et al. 2015)
Adolescents (11-18/19 yrs)	NCT01423084	Australia and Canada	344	11-17 years	4CMenB	2 doses of 4CMenB 2 months apart	Immunogenicity by hSBA assay, lot to lot consistency, safety profile.	hSBA titres of >5 against fHbp, NadA and PorA reference strains observed in $>90\%$ participants two weeks after dose 2. Minimal reduction in hSBA activity seen after one month. Both lots consistent with acceptable safety profile.	(Perrett et al. 2015)
	NCT00661713	Chile	1631	11-17 years	4CMenB	1, 2 or 3 doses at 1, 2 or 6 month intervals.	hSBA after 1, 2 or 3 doses measured at baseline and one month following vaccination.	99-100% participants has $>1:4$ hSBA titre after 2 or 3 doses vs 91-97% of those receiving 1 dose. 91-100% of those receiving 2 or 3 doses had $>1:4$ titres after six months vs 73-76% after 1 dose.	(Santolaya et al. 2012)
Adults ($\geq 18/19$ yrs)	NCT00560313	Germany and Italy	54	18-50 years	4CMenB or Men ACWY-CRM	4CMenB at baseline, 2 and 6 months followed by MenACWY-CRM at 7 months.	hSBA titre assessed 1 month post-vaccination. Safety and tolerability.	$\geq 80\%$, $\geq 91\%$ and $\geq 92\%$ of vaccinees achieved a hSBA titre of $\geq 1:4$ after dose 1, 2 and 3, respectively. Acceptable rates of adverse events/reactions, however, greater than with MenACWY-CRM.	(Kimura et al. 2011)

In 2012 and 2013, 4CMenB was licenced under the trade name Bexsero® for use in those aged over two months in Europe and Australia, respectively. It was also licenced in Canada in late 2013 for those aged from two months to 17 years. In January 2015, 4CMenB gained FDA accelerated approval in the US for use in those aged 10-25 years. In 2015, the UK became the first country to introduce 4CMenB into the national infant immunisation schedule. The vaccine was introduced for newborn infants from September 2015 at a 2 and 4 month primary schedule with a 12 months booster dose (Ladhani et al., 2015b). A small catchup campaign was also launched for infants born from 1st May 2015. These infants received one or two doses of 4CMenB with their 3 month and/or 4 month routine vaccinations depending on their age, as well as the 12 month booster. A single dose of paracetamol has been shown to reduce the incidence of fever without impacting on immunogenicity (Prymula et al., 2014) and paracetamol is recommended following primary vaccination with 4CMenB and at 4-6 hourly intervals as required (Ladhani et al., 2015b).

In the first ten months of the programme, routine uptake of 4CMenB in infants was high (95.5% for one dose; 88.6% for two doses) and a 50% reduction in incidence rate ratio was observed in the vaccine-eligible cohort (Parikh et al., 2016). A vaccine effectiveness of 94.2% was calculated based on a estimated strain coverage of 88% (Frosi et al., 2013). Subsequent analyses of more recent strains have revised down the strain coverage estimate for England, Wales and Northern Ireland suggesting that vaccine effectiveness is likely to be higher than 95% for this period (Parikh et al., 2017).

1.8.5.2 rLP2086

rLP2086 is a recombinant vaccine composed of two lipidated fHbp peptides. Selection of the two variants was based on the observed cross-reactivity within fHbp subfamily A (variant groups 2 and 3) and B (variant group 1) but the limited reactivity between the subfamilies (Pizza et al., 2008; Fletcher et al., 2004). Consequently, including one variant from each subfamily should theoretically provide protection against all fHbp variants. The vaccine contains variant 1.55 (B01) and 3.45 (A05) (Zlotnick et al., 2015). Early data suggested that lipidated fHbp is more likely to induce SBA than the corresponding non-lipidated peptide (Fletcher et al., 2004). In pre-clinical studies using

rabbit serum, rLP2086 elicited SBA against 87 of 100 diverse isolates. The immune serum was shown also to be reactive against fHbp on the cell surface in a subfamily specific manner. Expression of fHbp (measured using monoclonal antibody MN86-994-11-1) correlates positively with killing using rLP2086 anti-serum in the SBA assay (Jiang et al., 2010).

Early clinical trials were carried out using 3-dose schedules (0, 1 and 6 or 0, 2 and 6 months) of varying vaccine concentrations and hSBAs were performed against at least 4 test strains which had been selected to represent a disparate, heterologous fHbp peptides expressed at low/medium levels (Zlotnick et al., 2015). A phase I study in toddlers (18-36 months) found acceptable tolerability to the vaccine and high hSBA titres against a strain expressing a homologous fHbp variant (3.45; A05) but seroconversion (≥ 4 -fold increase in hSBA titre) was seen in $< 50\%$ of vaccinees after dose 3 when tested against three of the four initially-selected strains expressing heterologous fHbp variants, even at the highest vaccine dose (200 μg) (Marshall et al., 2012). Similar results were seen in older children (8-14 years) (Nissen et al., 2013). SBA responses in adolescents and adults were, however, much more robust and, after 3 doses of 60 μg , 120 μg or 200 μg , seroconversion or titres of ≥ 4 were observed in $\geq 90\%$ of participants against strains with diverse fHbp variants (Richmond et al., 2012a, 2012b). The vaccine exhibits an acceptable safety profile in this age group (Sheldon et al., 2012).

Based on these early data, a final formulation of 120 μg (60 μg of each lipoprotein) was selected and confirmed to be tolerated and immunogenic in adolescents and adults (Marshall et al., 2013; Ostergaard et al., 2016). A subsequent study in infants was, however, terminated early due to safety concerns. Following a single dose of 60 μg , 80% of participants reported mild/moderate fevers (Martinon-Torres et al., 2014). These findings have precluded further trials of rLP2086 in infants.

A large, phase III trial in adolescents compared rLP2086 vaccine schedules and observed that whilst three doses provided higher hSBA titres, the majority of participants generated titres of ≥ 8 against all four test strains after only two doses (Vesikari et al., 2016). They also found that SBA responses positively correlate with the

amount of time between doses. These findings are confirmed by a subsequent Phase III study in adolescents and young adults in which, against most test strains, three doses elicited significantly greater SBA conversion than after two doses (Ostergaard et al., 2017). A follow-up to a previous phase II trial in adolescents suggested that protection may last up to four years in many cases. Protective hSBA titres (≥ 4) against 3 of the 4 test strains were observed in >50% of vaccinees 48 months after the third rLP2086 dose (Marshall et al., 2017). Conversely, a small study in laboratory workers immunised with rLP2086 found that hSBA titres waned considerably 11 months after the third dose (Lujan et al., 2017).

rLP2086 was licenced in the US in 2014 under the trade name Trumenba®. The vaccine was accepted for use in 10-25 year-olds via the FDA accelerated approval programme. The vaccine was also licenced in Europe in 2017 for use in those aged ten years and older. As the vaccine is restricted to use in adolescents/ adults, the potential utility of the vaccine is likely to be lower than that of 4CMenB, which can be used to directly protect infants. rLP2086 may, however, be useful for controlling sporadic outbreaks in older populations (e.g. universities), especially where the circulating invasive strain may not be covered by 4CMenB antigens (Soeters et al., 2017b). Like 4CMenB, routine use of rLP2086 in adolescents is unlikely to be cost-effective unless the vaccine is shown to impact the acquisition of nasopharyngeal carriage, which can lead to indirect herd protection of younger populations as observed with group C conjugate vaccines. Limited data are available on this question; however a recent carriage study in a US university observed no reduction in carriage following vaccination with rLP2086. (Soeters et al., 2017a). This study involved relatively small numbers of participants and so additional, suitably-powered studies are required in order to conclusively determine the impact of this vaccine on carriage.

1.8.6 Predicting Vaccine Strain Coverage and Assessing Impact

Predicting the strain coverage of vaccines consisting of capsular polysaccharide antigens, which have a relatively uniform composition, is fairly simple as polysaccharide vaccines will protect against all strains belonging to the corresponding capsular group. Such protection is dependent upon adequate capsular expression of

the polysaccharide, however, as an indispensable virulence factor, the vast majority of invasive strains express the antigen in abundance (Gray et al., 2006).

In contrast, many protein antigens, particularly those under immune selective pressure, exhibit significant sequence variation which can restrict the breadth of the bactericidal response elicited by protein-based vaccines (Urwin et al., 2002). Additionally, many antigens can be relatively sparse on the outer membrane surface and the level of expression of protein antigens (e.g. fHbp) can vary widely between strains (Biagini et al., 2016). As such, strain coverage predictions of novel recombinant vaccines require a much more complex evaluation of antibody cross-reactivity against the specific peptide variants in conjunction with measurements of surface expression by individual invasive strains.

1.8.6.1 Predicting the strain coverage of 4CMenB

The multi-component nature of 4CMenB complicates the assessment of its strain coverage. During its licensure, a small panel of strains was used in the SBA to assess the immunogenicity of each antigenic component. Using the SBA assay against the large numbers of strains required for an accurate prediction of strain coverage would be time consuming and would require large amounts of immune serum and exogenous complement. In order to facilitate such large-scale strain coverage evaluations, the Meningococcal Antigen Typing System (MATs) was developed. The purpose of the MATs is to predict killing of strains in the hSBA assay by antibodies elicited by 4CMenB.

The MATs uses a sandwich-ELISA format to measure the binding of polyclonal rabbit antibodies, each raised against the individual recombinant 4CMenB antigens (fHbp, NadA and NHBA), to the corresponding antigens present in the lysates of each strain tested. The amount of antigen bound to the wells of the plate is a function of the cross-reactivity between the harboured antigenic variant and the antibodies, as well as the amount of antigen present in the lysate. The signal intensities are compared to those of reference strains to produce a relative potency (RP) value for each antigen. A MATs assessment study by Donnelly et al. established RP cut off values for each antigen, termed positive bactericidal thresholds (PBT), and found that strains with RPs above the assigned PBT for one of the three antigen had >80% likelihood of being killed by

pooled serum from infants who have received 4CMenB at 2, 3, 4 and 12 months (Donnelly et al 2009). As the expression level of the PorA OMV antigen is not a determinant of immune recognition and strain killing, this component is not assessed using the ELISA. Typing of PorA is sufficient and the presence of the P1.4 VR2 epitope is predictive of strain killing by post-vaccination sera (Martin et al., 2006). As a result, for a strain to be predicted as 'covered' by the MATS, it must produce an RP above the assigned PBT for at least one recombinant antigen and/or possess the PorA P1.4 VR2 epitope.

The MATS assay has been established within many reference units throughout Europe and beyond. The first large-scale use of the MATS was in a Europe-wide assessment of 1052 invasive group B isolates collected during the 2007/08 epidemiological year from five countries. The study reported an overall European strain coverage of 78% (73%-87% inter-country variation) with 73% of English and Welsh strains covered (Vogel et al., 2013). FHbp and NHBA were the predominant antigenic contributors with the majority of all tested European isolates MATS positive for these one or both of these antigens. Only around 7% of strains were MATS positive for NadA. In England and Wales, 69% of covered strains were positive for >1 antigen (Vogel et al., 2013).

In 2016, a comparative analysis of the 2007/08 English and Welsh isolate data and MATS data from group B isolates collected during the 2014/15 epidemiological year reported an small but significant reduction in strain coverage between these two years (to 68% in 2014/15) (Parikh et al 2017). Analysis showed this decrease in coverage was due to a reduction in a predominant, cross-reactive NHBA variant (peptide 21) driven by changes in CC distribution, particularly within the ST-269 complex (Parikh et al 2017). A number of other MATS assessments have been carried out in Europe and elsewhere (Bettinger et al., 2014; Tzanakaki et al., 2014; Medini et al., 2015; Abad et al., 2016). As in the UK, differences in strain coverage across these countries were also attributed to natural variation in the geographical CC distribution demonstrating that strain coverage of protein-based vaccines can be sensitive to changes in the antigenic distribution within the hyper-virulent strain population.

Two recent studies have compared MATS results to SBA killing and both concluded that MATS provides a conservative estimation of coverage as a small number of MATS-negative strains were killed by pooled infant and adolescent immune serum in the SBA assay. It has been suggested that, in the SBA (and *in vivo*), the immune response may be augmented by synergism between the antibodies targeting different antigens, an effect that would not be observed in the MATS. Furthermore, the MATS would not take into account bactericidal activity of antibodies targeting additional, minor antigens with the OMV preparation (Abad et al., 2015; Frosi et al., 2013).

MATS is also useful for investigations into potential vaccine failures. Invasive isolates from 4CMenB vaccinees are tested to determine whether the strain should have been killed by immune serum. In cases in which a viable isolate has not been obtained, predictions of vaccine strain coverage are based on genotypic information only. In these cases a strain can only be considered covered if it possesses PorA P1.4 and/or one of a small number of fHbp variants which have been shown to be reliably MATS positive in isolates. These are peptide variants 1.1, 1.4, 1.37, or 1.232 (Vogel et al., 2013).

1.8.6.2 Predicting the strain coverage of rLP2086

As rLP2086 is composed solely of two fHbp variants, predicting strain coverage is relatively less complex. During the licensure of rLP2086, the manufacturers demonstrated the ability of the vaccine to protect against strains expressing diverse fHbp variants. Thus, cross-reactivity between rLP2086-elicited antibodies and the fHbp variant harboured by an invading strain is assumed providing fHbp is expressed sufficiently on the surface of the bacterium. Thus, the characterisation of the specific fHbp variants harboured by invasive strains is not explicitly required to predict coverage, only evidence of fHbp surface expression. To this end, a flow cytometry assay named the Meningococcal Antigen Surface Expression (MEASURE) assay was developed in order to quantify the level of surface expression on meningococcal cells fixed using formaldehyde (McNeil et al., 2018).

The MEASURE assay utilises a monoclonal antibody (MN86-994-11-1) that recognises diverse fHbp peptide variants (of both subfamilies/all variant groups). In 2018, McNeil

et al. elucidated the binding epitope of MN86-994-11-1 on fHbp using hydrogen-deuterium exchange mass spectrometry. They showed that the antibody binds primarily to the C-terminal β -barrel. Residues 180 to 198 were of particular importance as they are involved in binding to the antibody among protein variants of both subfamilies. The binding affinities of the antibody were shown to be relatively consistent across eight different fHbp variants (McNeil et al., 2018).

The MEASURE readout is the mean fluorescence intensity (MFI) and directly correlates with the expression of fHbp on the cell surface. In a comparison of MEASURE results and the hSBA killing result (killing defined as 4-fold SBA titre rise) among 45 strains, expression levels of fHbp were positively correlated with the likelihood of killing by post-immunisation human serum (Jiang et al., 2010). McNeil et al. later confirmed these findings with an additional 109 strains. Whilst a poor correlation between the hSBA titre and MEASURE MFI was found, the MFI was predictive of killing overall. The data suggested that strains producing an MFI of 1000 (purported to be equivalent to 30 pg of fHbp / μ g of total cellular protein) or above have a 91% chance of susceptibility to anti-rLP2086 serum (McNeil et al., 2018).

1.9 IMD Surveillance in England and Wales

In England and Wales (E&W), national surveillance of IMD is carried out primarily by the Public Health England's Meningococcal Reference Unit (PHE MRU). PHE MRU carries out laboratory IMD confirmation and strain characterisation of clinical isolates submitted by microbiology laboratories throughout the E&W. Speciation through oxidase, catalase and carbohydrate utilisation tests are typically followed by phenotypic finotyping using Dot-blot ELISA and/or co-agglutination (Gray et al., 2006).

Following phenotypic characterisation, meningococcal isolates undergo whole genome sequencing. All meningococcal isolates received since July 2010 have been (or are due to be) whole genome sequenced using the Illumina HiSeq platform. The assembled genomic data are stored in the MRF MGL within BIGSdb hosted by the University of Oxford (www.pubMLST.org) (Jolley and Maiden, 2010). The database features automated indexing tools which search for and characterise known meningococcal genes within the uploaded data, assigning arbitrary numeric identifiers to each unique allele.

In addition to isolate characterisation, a real-time PCR screening assay, targeting the meningococcal capsular gene *ctrA*, has been used to test submitted clinical specimens since 1997. Figure 1-5 illustrates the numbers of cases confirmed by each confirmation method per year since 1998/99.

Within two years of the introduction of the PCR diagnostic service, a 56% increase in confirmed disease cases was observed (Guiver and Borrow, 2001). In recent years just less than half of IMD cases (44.9%) are confirmed solely through PCR analysis of culture-negative clinical samples and approximately a quarter by both culture and non-culture (Figure 1-5) (Heinsbroek et al., 2013). Taqman® assays targeting *siaD* (for groups B, C, Y and W) and *mynA* (for group A) are also used to allow genogroup determination (Hackett et al., 2002a).

The assay was originally a triplex assay designed to also detect *Haemophilus influenzae* (*bexA*) and *Streptococcus pneumoniae* (*ply*) DNA. In 2003, an additional meningococcal reverse primer was included in order to compensate for a primer site mismatch which led to a lack of coverage of strains belonging to the ST-269 cluster (ST-269 complex) (Gray et al., 2012).

In 2011, the assay was again amended, removing the *Haemophilus* primers/probes (due to low numbers of cases) and incorporating the group B-specific (*siaDb*) target within a lyophilised master mix format. As group B is the predominant capsular group in E&W, it was reasoned that this change would, in most cases, eliminate the requirement of a secondary PCR for group confirmation thereby reducing the assay turnaround times (McHugh et al., 2015). Due to a detected reduction in the sensitivity of the assay, the mix was changed again in 2015 to a 'wet mix' with *ctrA*-specific ABY dye probe (unpublished data, PHE MRU).

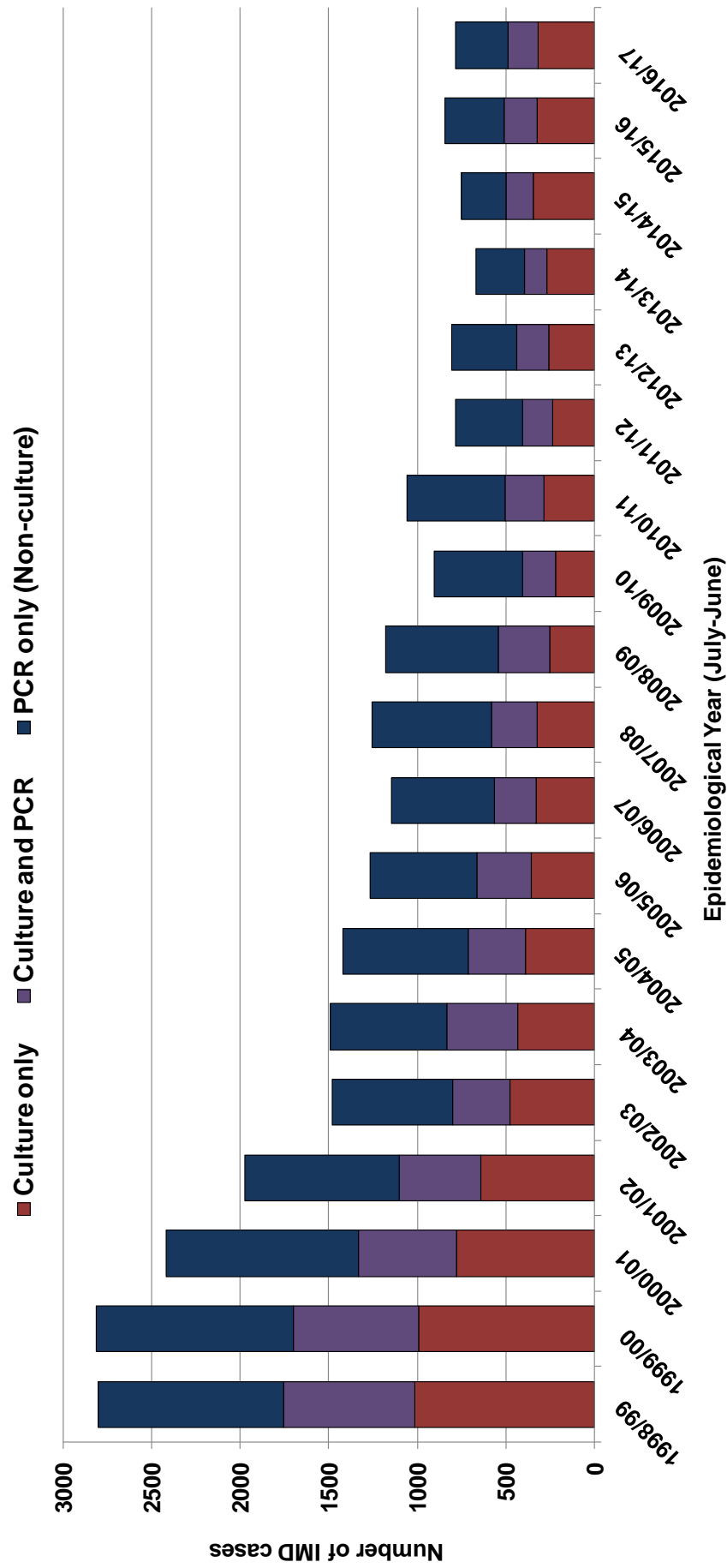


Figure 1-5: Number of IMD cases confirmed by PHE MRU from 1998/99 to 2016/17 by method of confirmation. A gradual reduction in total IMD cases was observed. Of all cases confirmed over the nineteen epidemiological years, 44.9% were confirmed by PCR alone. The lack of a viable isolate in these cases limits the extent of strain characterisation.

Due the dearth of meningococcal DNA and the presence of human DNA among clinical specimens, whole genome sequencing of meningococci directly from these specimens is not yet possible. Consequently, beyond capsular grouping, non-culture strain characterisation is limited to sequencing of the *porA* gene (not published) and the seven MLST loci (Birtles et al., 2005) using nested PCR assays, however, the MLST assay is not routinely used due to greater number of targets involved.

Following the introduction of 4CMenB into the routine infant immunisation programme in 2015, PHE implemented an Enhanced Surveillance programme to monitor the impact of this intervention and other meningococcal vaccine programmes (i.e. MenC and MenACWY) in terms of measuring vaccine effectiveness and assessing potential changes in strain distribution. Clinicians are asked to provide additional EDTA samples and throat swabs from suspected IMD cases. These are sent to PHE MRU and can be used for genotypic strain characterisation and/or future seroprevalence studies. The limited strain characterisation possible for non-culture cases impedes an accurate assessment of the impact of 4CMenB introduction on the meningococcal strains in circulation in England and Wales as well as hindering the accuracy of strain coverage predictions for other non-capsular vaccines.

Thesis Objectives and Structure

The overall aim of the research was to develop and introduce a number of assays/techniques designed to improve and/or increase the phenotypic and genotypic data generated by PHE MRU prior to and as part of PHE's Enhanced Surveillance programme.

The primary objectives were to:

1. Develop a non-culture PCR sequencing assay to allow amplification and characterisation of the *fHbp* allele directly from clinical specimens submitted to PHE MRU.
2. Apply this *fHbp* genotyping assay to five years (2011-2015) of submitted clinical specimens in order to reveal the distribution of fHbp variants among non-culture strains and assess any disparity with that of corresponding invasive isolates.
3. Assess the effectiveness of the Agilent SureSelect XT system for enrichment of meningococcal DNA within non-culture DNA extracts in order to facilitate whole genome sequencing.
4. Transfer an established flow cytometry-based assay designed to quantify the level of fHbp surface expression and perform inter-laboratory comparisons of results.

2.0 METHODS AND MATERIALS

2.1 Storage and culturing of *N. meningitidis* isolates

2.1.1 Storage of meningococcal isolates

All meningococcal isolates used were stored at -80 °C on Microbank® beads (Prolab Diagnostics, Canada) or equivalent. These porous beads provide a solid support for bacterial storage. To inoculate a vial of beads for bacterial storage, an overnight culture of the required strain was prepared. A 10 µL loop containing bacterial growth was added to the vial and resuspended in the glycerol broth by rotating the loop. The lid was then replaced and the vial was inverted to mix the beads within the broth. The broth was then removed and discarded using a plastic pipette. The bead vial was then stored at -80°C.

2.1.2 Culturing meningococci on agar plates

All bacterial manipulations were performed within a class I microbiological safety cabinet (MSC, Contained Air Solutions, UK) on Columbia Blood Agar (CBA) (Oxoid, UK) unless otherwise stated. Bacteria were applied to the agar using 10 µL sterile plastic loops (from Microbank® beads) or using a pipette (broth/liquid) and streaked for single colony isolation. Agar plates were allowed to dry within the MSC before being transported to a CO₂ incubator (LEEC, UK) for overnight incubation (16-20 hours) at 37 °C with 5% (v/v) CO₂.

2.1.3 Preparation of meningococcal liquid suspensions

Preparation of meningococcal suspensions within broth was achieved by transferring bacteria from agar plates grown overnight directly to the broth (see 2.1.2). Within the MSC, a sterile cotton or polyester swab was used to remove the required number of meningococcal colonies for the agar surface. The swabs were then placed into the liquid and gently agitated along the inside of the vessel to remove the colonies and break up the cells. Once a suspension of a sufficient concentration was achieved, the swab was discarded and the liquid vessel sealed.

2.2 Storage and handling of clinical specimens

2.2.1 Storage of clinical specimens

All clinical specimens (e.g. EDTA blood, CSF, knee fluid, pericardial fluid) were stored at -80°C in the original vials received by PHE MRU.

2.2.2 Handling of clinical specimens

Clinical specimens were treated as potentially infectious and were manipulated within an MSC.

2.3 Preparation, quantification and storage of DNA extracts

2.3.1 Extraction of DNA from meningococcal isolates

Extraction of DNA from meningococcal isolates was performed by PHE MRU as part of previous sequencing projects (Hill et al., 2015; Vogel et al., 2013). The isolates were extracted using the DNeasy Blood and Tissue kit (Qiagen, UK) following the manufacturers' gram-negative bacteria protocol (DNeasy® Blood & Tissue Handbook, July 2006).

2.3.2 Extraction of DNA from clinical specimens

Unless otherwise stated, DNA extraction from clinical specimens was performed by PHE MRU as part of routine diagnostic PCR testing. PHE MRU uses the MDx biorobot platform (Qiagen, UK) in accordance with the manufacturer's protocol with a final elution volume of 80 µL.

Alternatively, DNA extraction from clinical specimens was performed manually using DNeasy Blood and Tissue Kit following the 'Animal Blood' protocol (DNeasy® Blood & Tissue Handbook, July 2006). The DNeasy protocol involved adding 100 µL of specimen to a 1.5 mL screw-capped tube containing 20 µL of Proteinase K solution (Qiagen, UK) before adding 100 µL of phosphate-buffered saline (PBS, ThermoFisher, US). Then, 200 µL of Buffer AL (Qiagen, UK) was added and the suspension was vortexed vigorously. The tube was incubated at 56 °C on a heating block (Grant Instruments, UK) for 10

minutes. The tube was then pulse spun in a Micromax (IEC) or Biofuge Pico (Heraeus) microfuge in order to remove suspension from the tube lid before the addition of 200 μ L of Ethanol (>99.5%, Sigma Aldrich, UK) to the suspension. The tube was once again vortexed and centrifuged to remove suspension from the lid. The tube contents were then transferred to a DNeasy spin column within a 2 mL collection microtube (Qiagen, UK). The spin column and collection microtube were spun at 6000 x g for 1 minute to draw the lysate through the filter. The column was then carefully removed and placed into an empty collection microtube. To wash the filter, 500 μ L of Buffer AW1 was added to the spin column and spun once again at 6000 x g for 1 minute. The spin column was added to another empty collection microtube before the addition of 500 μ L of Buffer AW2 to the column and centrifugation at 16,000 x g for 3 minutes. The spin column was then placed into a sterile Eppendorf tube for elution. The elution was performed by adding 50 μ L of Buffer AE (Qiagen, UK) to the spin column and allowing one minute incubation at RT. The column was then spun at 6000 x g for one minute. This was then repeated within another 50 μ L of Buffer AE giving a total elution volume of 100 μ L. The use of two successive elutions maximised the DNA yield whilst minimising the volume in which the DNA was eluted.

2.3.3 Quantification of DNA

DNA quantification was performed using a Qubit 2.0 fluorometer in conjunction with the Qubit® double-stranded DNA (dsDNA) BR Assay Kit (ThermoFisher, US). Prior to use, calibration of the fluorometer was performed using two standards provided in the kit. To calibrate, a sufficient volume of Qubit® dsDNA BR Reagent was diluted in Qubit® dsDNA BR Buffer at a 1:200 concentration. For each standard, 190 μ L of diluted reagent was added to a thin-wall, clear, 0.5 mL PCR tube (Corning, US) before adding 10 μ L of the standard. The standards were incubated for 1 minute at room temperature (RT) before being read in the fluorometer.

For each extract to be quantified, 190 μ L of diluted Qubit® dsDNA BR Reagent was added to a PCR tube before adding 5 μ L of the DNA extract and incubating for 1 minute at RT. The tubes were then placed into the fluorometer and read by pressing 'READ'.

The assay concentration (diluted concentration) and the stock concentration (extract concentration) were recorded for each extract.

2.3.4 Collation of non-culture DNA extracts for *fHbp* genotyping

Attempts were made to obtain DNA extracts from all non-culture cases confirmed by the PHE MRU from 1st January 2011 to 31st December 2015. For cases in which there was no available extract, re-extraction of DNA from the original specimen was carried out either manually using Qiagen Blood and Tissue DNeasy kit (see section 2.3.2). Cases in which no specimen and/or extract could be obtained could not be tested.

2.3.5 Storage of DNA extracts

All DNA extracts were stored in screw-capped microfuge tubes or Eppendorf tubes at 4 °C or -80 °C.

2.4 Polymerase chain reaction

2.4.1 Primer preparation and storage

All primers were produced by Eurofins Genomics using HPSF (High Purity Salt Free, a proprietary reverse phase cartridge purification method) at a 0.20 µmol synthesis scale. Primers were supplied lyophilised and reconstituted in molecular grade water (Sigma Aldrich, UK) to a concentration of 100 µM (master stock, x20). To prepare working stocks (5 µM), 50 µL of the master stock was diluted in 950 µL of molecular grade water (Sigma Aldrich, UK) and mixed. All primer stocks were stored at -20 °C.

2.4.2 Preparation of PCR reaction master mix

All PCR master mixes were prepared using the HotStarTaq DNA polymerase kit (containing HotStarTaq DNA polymerase, 10x PCR Buffer and 25 mM MgCl₂) and dNTP mix (Qiagen, UK). All reagents were stored at -20 °C. To prepare a master mix, PCR reagents and required primers were removed from storage and allowed to defrost at RT. Reagents were then mixed by inverting the tubes and then spun in microfuge to remove reagent from tube caps. Master mixes were prepared within a PCR cabinet by

adding the required volumes of each reagent to a 1.5 µL screw-capped micro tubes or 30 mL Universal tube and gently mixing using a pipette (Table 2-1).

Table 2-1: Standard PCR master mix reagent volumes for PCR reactions

Reagent	Volume per 25 µL reaction (µL)	Volume per 50 µL reaction (µL)	Volume per 100 µL reaction (µL)
HotStarTaq PCR buffer	2.5	5	10
HotStarTaq DNA Polymerase	0.125	0.25	0.5
dNTPs (10 mM each)	0.5	1	2
PCR primer 1 (5 µM)	2.5	5	10
PCR primer 2 (5 µM)	2.5	5	10
MgCl ₂ (25 mM)	variable*	variable*	variable*
Distilled water	variable#	variable#	variable#

*added to the required concentration. #added up to required master mix volume (reaction volume – extract volume)

The required volume of master mix was added to the wells of a MicroAmp® Optical 96-Well Reaction Plate (ThermoFisher, US).

2.4.3 Addition of DNA extracts and use of thermal cycler.

The DNA extracts were defrosted at RT (if frozen) before being spun briefly in the centrifuge to clear the tube lids. The required volume of DNA extract was then added to the appropriate well of the PCR reaction plate containing the master mix. The plate was sealed using MicroAmp® Optical 8-Cap strips (ThermoFisher, US). The plate was placed onto a Verity thermal cycler (Applied Biosystems, US) and the required PCR programme was completed.

2.4.4 Gel electrophoresis of PCR products

2.4.4.1 Preparation of agarose gels.

The presence/absence of amplified products was determined through visualisation on 2% agarose gels. Agarose gels were prepared by weighing 3 g of agarose (Sigma Aldrich, UK) and suspending it in 150 mL of 1x Tris-Borate EDTA (TBE) buffer (89 mM Tris-borate

and 2 mM EDTA, pH 8.3., Sigma Aldrich, UK). The suspension was heated in a microwave oven for up to two minutes until the agarose had fully dissolved. After two minutes of cooling at RT, 15 µL of 10,000x SYBR® Safe DNA stain (Thermo Fisher, US) was added to the dissolved agar and mixed through gentle agitation. The agar was then poured into a casting tray left at RT until fully solidified. The gel was placed into a gel tank (ThermoFisher, US) and submerged in 1x TBE buffer.

2.4.4.2 Preparation of gel loading buffer.

A 30% (v/v) glycerol solution was prepared by mixing the appropriate amount of glycerol to distilled water. Small amounts of Bromophenol blue (Sigma Aldrich, UK) were then successively added until the buffer was stained to a dark green colour.

2.4.4.3 Loading and running of agarose gels

A volume of 5 µL of loading buffer was added to 5 µL of each PCR product and loaded into the the agarose gel. For each line of wells in the gel (i.e. each gel comb), 6 µL of GelPilot 100 bp plus molecular ladder (ML, Qiagen, UK) was loaded.

The gel was run at 100 V. The length of electrophoresis time required depended on the intended PCR product length. For the *fHbp* PCR round-two product (~1330-1530 bp), approximately 15 minutes was sufficient.

2.4.4.4 Visualising PCR products in gel

After electrophoresis, the gel visualised using the G:BOX transilluminator (Syngene, US). Using the Genesys software, the gel image was captured using the automated capture settings. The brightness and contrast of the image was adjusted as appropriate post-capture and the image was cropped to include the used wells only.

The intensity of the PCR product bands was determined visually in comparison with the molecular ladder to assign approximate band intensities: strong, medium, weak or very weak. The intensity was recorded and would determine the volume of water added to the products in the subsequent PCR clean up stage.

2.4.5 Clean-up of PCR products

PCR products were cleaned using ExoSAP-IT® (Affymetrix, US). The product contains Shrimp Alkaline Phosphatase and Exonuclease I, two hydrolytic enzymes which digest residual dNTPs and primers/single stranded DNA remaining within the reaction mix.

PCR cleanups were performed in MicroAmp® fast optical 96-well reaction plates (Thermo Fisher, US). For each sample, 2 µL of ExoSAP-IT® was added to 5 µL of each PCR reaction mix. The plate was heated on a ABI 9800 Fast thermal cycler (Applied Biosystems, US) using the following protocol: 37 °C for 15 minutes, 80 °C for 15 minutes, retained at 4 °C.

The clean-up mix was then diluted using molecular grade water. The dilution factor used for each product was dependent upon the intensity of the corresponding band produced on the agarose gel. 'Strong' bands were diluted ¼, medium bands were diluted ⅓ and weak bands diluted ½. To very weak products, 3 µL of water was added (1/1.42 dilution).

2.5 Optimisation and analysis of *fHbp* PCR assay

2.5.1 Primer selection and design

Selection of PCR and sequencing primers was primarily based on the location of its complementary sequence in relation to *fHbp* and the conservation of the nucleotide sequence among diverse meningococcal strains. To identify new primer candidates, the Primer Quest online tool (Integrated DNA Technologies, US <https://www.idtdna.com/Primerquest/Home/Index>) was used to identify putative candidates within input sequences. To identify suitable PCR primer-sites, DNA sequences of the flanking regions of *fHbp* among 15 complete meningococcal genomes from the Genbank database (National Center for Biotechnology Information (NCBI), US) were inputted into Primer Quest (Appendix I).

The suitability of parameters such as melting temperature, percentage G/C content (%GC) and the likelihood of hairpin/dimer formation were also assessed using the online Oligoanalyzer tool (Integrated DNA Technologies, US).

<https://www.idtdna.com/calc/analyzer>). Melting temperatures of ~55-60 °C and %G/C of 40-60% were considered ideal. Primers which form internal hairpins and self-dimerisations with delta G score of <-9 kcal/mole were considered unsuitable (as recommended by Integrated DNA Technologies, US).

To assess location and conservation of primers, DNA sequences of *fHbp*, the flanking genes (GNA1869 and GNA1871 in strain MC58) and intergenic regions from 392 invasive isolates (Appendix I) were downloaded from genomic data held within the PubMLST database (University of Oxford, <http://pubmlst.org/neisseria/>). Sequences were aligned using BioEdit Sequence Alignment Editor version 7 (Hall, 1999). Further assessment of potential non-specific binding within the target region was performed following deletion of the corresponding primer-binding sites from sequence alignments using Sequencher v4.7 (Gene Codes Corporation, Ann Arbor, MI USA). Primer candidates with significant complementarity (≥ 4 bp) to other targets were not selected.

2.5.2 Primer optimisation

Optimisation of new primer sets was carried out empirically by varying the master mix MgCl_2 concentration as well as the thermal cycler annealing temperature, extension time and PCR cycle number. Conditions which yielded the strongest/brightest product band of appropriate size and the least visible non-specific amplification (on 2% agarose gel) were selected. Optimisation was performed using single PCR rounds (i.e. not nested). Other than specific variable parameters (annealing temperature, extension time and cycle number), which were selected during optimisation, standard thermal cycler conditions (as recommended in Qiagen HotStarTaq PCR Handbook (October 2010)) were used. These included 95°C for denaturation (15 mins initially and then 1 minute per cycle), an annealing time of 30 secs, an extension temperature of 72°C and a final extension step of 4 mins at 72°C. For each primer set, a range of annealing temperatures encompassing the predicted primer melting temperatures was chosen for testing. Initial extension times were calculated assuming one minute per 1000 bp of product was sufficient for amplification. A PCR cycle number of 35 was initially used and adjusted as appropriate.

DNA extracts from seven meningococcal isolates were selected for use as template during assay development. These are listed in Appendix II. The use of purified meningococcal DNA allowed sufficient amplification after only one PCR round (i.e. non-nested) to allow visualization on an agarose gel. The isolate panel possessed fHbp variants from different variant groups/modular groups. Four of the seven were used for PCR primer optimisation (highlighted).

2.5.3 Assessment of *fHbp* PCR assay sensitivity

2.5.3.1 Preparation of diluted extract series

The assessment of assay sensitivity was carried out through analysis of pre-quantified DNA extracts. Extracted DNA from each of the isolates listed in Appendix II was quantified and adjusted in elution buffer (Buffer AE, Qiagen, UK) to a concentration of 6 ng/mL. For each adjusted extract, a series of eight 10-fold dilutions was carried out by transferring 20 μ L of each successive diluted extract to 180 μ L of elution buffer.

2.5.3.2 Calculation of molecular weight of meningococcal genome

In order to determine the assay sensitivity in terms of genome copies, a calculation was performed to estimate the molecular weight of an average meningococcal genome. The calculation assumed a single base pair of dsDNA weighs 650 Da and the average meningococcal genome length is 2.2 million bp (Mbp). Following these assumptions:

$650 \text{ Da} \times 2,200,000 = 1.43 \times 10^9 \text{ Da} = 1.43 \times 10^9 \text{ g/mole.}$
 $1.43 \times 10^9 \text{ g of meningococcal DNA contains } 6.022 \times 10^{23} \text{ genome copies.}$
 $6.022 \times 10^{23} / 1.43 \times 10^9 =$
 $4.21 \times 10^{14} \text{ copies per g.}$
 $4.21 \times 10^{11} \text{ copies per mg}$
 $4.21 \times 10^8 \text{ copies per } \mu\text{g}$
 $421,000 \text{ copies per ng}$
 $421 \text{ copies per pg}$
 $0.421 \text{ copies per fg}$
 $0.421 \times 10^{-3} \text{ copies per ag}$

These numbers were used to determine the number of genome copies per microlitre for each extract dilution. These figures were then used to estimate the numbers of copies per reaction by multiplying by the number of microlitres of DNA extract used in the reaction mix.

2.5.3.3 Analytical sensitivity assessment

The analytical sensitivity of the PCR assay was determined by identifying the highest dilution at which PCR products were visible on a 2% agarose gel. Assessment was carried out following PCR round one only and then following the nested PCR protocol. Initially, for PCR round one, 5 µL of each DNA extract was added to a 25 µL reaction. Products were visualised on a gel. For dilutions at which faint or no round one products were visible, different volumes (1 µL, 2 µL and 5 µL) of the round one mix were then used as template in 25 µL PCR round two reactions. Products were again visualised on a gel.

To assess the impact of the DNA extract volume used in the first round of a nested PCR assay on the chances of *fHbp* amplification, 5 µL, 10 µL and 20 µL of each 10^{-7} and 10^{-8} extract was added to PCR round one reactions with total volumes of 25 µL, 50 µL and 100 µL, respectively. The total reaction volumes were increased along with template volume to ensure the proportional amount of template added was consistent across each extract volume (i.e. 20% of reaction mix). Following round one, 2 µL of each round one mix was added to a 25 µL PCR round two reaction. Products were visualised on a gel.

2.5.4 *ctrA*-specific real-time PCR analysis of analytical sensitivity extracts

2.5.4.1 Standard meningococcal Taqman® assays

In order to provide a correlation between the estimated DNA sensitivity limit and the cycle threshold (Ct) value, the diluted extracts of the analytical sensitivity panel were tested using *ctrA*-directed real-time Taqman® assays (Thermo Scientific, US). In 2012, the duplex *ctrA/ply* Taqman® assay was replaced by a quadriplex, lyophilised master mix. In order to obtain the most accurate comparison, the dilution extract panel was tested using both assay formats. The lyophilised mix was supplied incorporating all four target components. Although the *ply* component of the wet-mix was not required, it was included in order to prevent any impact on the assay results.

2.5.4.2 Wet mix Taqman® assay

For the wet master mix assay, the reagents were removed from the freezer, allowed to defrost. For each of the two forward primers, 37.5 µL of the master stock (80 µM) was added 500 µL of molecular grade water (Sigma Aldrich, UK) in a screw-capped microfuge tube (Sarstedt, Germany). These two solutions were then mixed resulting in a pre-mix with a concentration of 3 µM of each forward primer. A pre-mix of the three reverse primers was similarly prepared by diluting from 80 µM master stock to a concentration of 3 µM of each reverse primer within 1 mL of molecular grade water. Finally a pre-mix of the two probes was prepared by diluting master stock (50 µM) to final concentration of 4 µM in 1 mL of water.

For each extract, 2.5 µL of each primer/probe pre-mix was added to 12.5 of Taqman® Fast Advanced Master Mix (Applied Biosystems, US). For each dilution extract, 5 µL was added to 20 µL of the master mix. Due to stochastic effects observed during the analytical sensitivity analysis, the two lowest dilutions (10^{-7} and 10^{-8}) of each isolate were tested in triplicate. The primer and probes sequences as well as the final working concentrations are shown in Table 2-2.

Table 2-2: Sequences and working concentrations of primers/probes within ‘wet’ mastermix duplex Taqman® assay.

Reagent	Primer/probe sequence (5'-3')	Concentration (nM)
<i>ctrA</i> forward primer	GCTGCGGTAGGTGGTTC	300
<i>ply</i> forward primer	TGCAGAGCGTCCTTTGGTCTAT	
<i>ctrA</i> reverse primer 1	TTGTCGCGGATTGCAACTA	300
<i>ctrA</i> reverse primer 2	TTGCCGCGGATTGGCCACCA	
<i>ply</i> reverse primer	CTCTTACTCGTGTTTCCAACCTGA	
<i>ctrA</i> Taqman® probe*	CATTGCCACGTGTCAGCTGCACAT	200
<i>ply</i> Taqman® probe#	TGGCGCCCATAAGCAACACTCGAA	

*- tetrachloro-6-carboxyfluorescein #- VIC reporter.

The PCR plate was sealed and placed on a 7500 Real-Time PCR System (Thermo Fisher, US). The PCR cycling parameters used were heating at 95°C for 10 minutes followed by

45 cycles of a two-stage temperature profile of 95°C for 15 seconds and 60°C for 1 minute.

2.5.4.3 Lyophilised Taqman® assay

The lyophilised Taqman® plates were manufactured by Applied Biosystems (US) and consisted of primers/probes targeting the *ctrA*, *ply* and *siaD* genes (Table 2-3).

Table 2-3: Sequences and working concentrations of primers/probes within lyophilised, quadruplex Taqman® assay.

Reagent	Primer/probe sequence (5'-3')	Concentration (nM)
<i>ctrA</i> forward primer	GCTGCGGTAGGTGGTTCAA	300
<i>ctrA</i> reverse primer 1	TTGTCGCGGATTTGCAACTA	300
<i>ctrA</i> reverse primer 2	TTGCCGCGGATTGGCCACCA	300
<i>ctrA</i> probe*	CATTGCCACGTGTCAGCTGCACAT	200
<i>ply</i> forward primer	TGCAGAGCGTCCTTTGGTCTAT	300
<i>ply</i> reverse primer	CTCTTACTCGTGGTTTCCAACCTGA	300
<i>ply</i> probe #	TGGCGCCCATAAGCAACACTCGAA	200
<i>siaDb</i> forward primer	ATTATACAGCCTGCTCATCTCTATATGC	900
<i>siaDb</i> reverse primer	TCCCTTCATCAATTAAATGAGTCGTA	900
<i>siaDb</i> probe α	TTACAGGCCACTACTCCT	300
Internal control forward primer	CCCTTGTCGAGCATTATAAAGAG	100
Internal control reverse primer	TTCATGTATGGTTCATCCTCGAA	100
Internal control probe≠	CATCGAGGCCAACTCGAAACATCGG	100

*- LIZ reporter, #- VIC reporter, α- tetrachloro-6-carboxyfluorescein (6-FAM) reporter, ≠-Cy5 reporter

For each well, the lyophilised mix was reconstituted in 20 µL of molecular-grade water before adding 5 µL of DNA extract. The PCR cycling parameters used were heating at 95°C for 2 minutes followed by 40 cycles of a two-stage temperature profile of 95°C for 3 seconds and 60°C for 30 seconds.

2.5.5 Specificity of *fHbp* PCR sequencing assay

2.5.5.1 Selection of meningococcal validation panel

To assess the specificity of the *fHbp* sequencing assay, a validation panel of 96 clinical non-culture specimens and corresponding isolates was compiled (i.e. matching isolate/specimen pairs from 96 clinical IMD cases). The validation panel was selected from IMD cases confirmed from 2010-2012. All the clinical isolates had previously

undergone whole genome sequencing. In order to select the most appropriate specimen/isolate pairs, the MLST (ST and CC) data from the isolates were downloaded from the MRF MGL within the BIGSdb database.

eBURST analysis (Feil et al., 2004) was used to predict the genetic relatedness of the isolates by dividing STs into groups based on the similarity of the seven allelic MLST variant combinations. All MLST profile data used in the analysis was obtained from the PubMLST database (accessed 30/12/2013). At least 6 identical MLST loci were required for group definition. In the analysis, Single-locus variants (SLVs) are grouped around a 'founder ST'. The founder ST has the most SLVs and is the putative ancestral ST for each group. Each group approximated the established meningococcal CCs. Isolates were selected based on the location of the corresponding STs on the eBURST diagrams. Disparate STs were selected within each ST group, and the number of isolates per group roughly correlated with the incidence of the corresponding CC within the genomic dataset.

For CC11, four of the six isolates were ST-11 and could not be meaningfully contrasted using conventional MLST. To achieve this, rMLST was performed on all available CC11 genomes within PubMLST using the BIGSdb Genome Comparator tool. Using the resultant distance matrix, a NeighborNet tree was constructed using SplitsTree v4 (Huson and Bryant, 2006). The isolates were highlighted on the tree in order to assess the genetic relatedness of the isolates among all ST-11 strains available.

As the DNA extracts from isolates contained DNA at a relatively higher concentration, all isolates were tested using PCR round one and two individually. This allowed for a more accurate assessment of the specificity of the primers. The corresponding non-culture extracts were tested using the optimised nested PCR protocol.

Amplification was confirmed using gel electrophoresis. Samples that failed to generate a PCR product of appropriate size were re-tested twice before being considered PCR-negative. To test the primer binding sequences for *fHbpRd2R*, the downstream *fHbp* flanking sequences among MRF MGL isolates from epidemiological years 2010/11 and 2011/12 (n=923) were downloaded from the PubMLST.org (via BLAST) and aligned in BioEdit v7 (Hall, 1999).

2.5.5.2 *Neisseria lactamica* isolates

In order to assess the wider conservation of the primers, six isolates of the related commensal *N. lactamica* were tested using the assay (Table 2-4).

Table 2-4: *N. lactamica* isolates used to assess primer site conservation

Isolate ID	Sequence Type	Clonal Complex
M98 250306	640	ST-640 complex
M00 240031	624	ST-624 complex
M03 240246	609	ST-613 complex (putative)
M98 250219	4192	Cluster 4192
M03 241253	9417	ST-613 complex
M99 242475	1494	ST-1494 complex

Extraction of the isolates was carried out as part of a previous study (Lucidarme et al., 2013a). The primer binding sequences from the isolate genomes were downloaded from PubMLST.org using the BLAST function. All *N. lactamica* isolates were tested in the two PCR rounds separately.

2.6 Optimised *fHbp* PCR parameters

2.6.1 Final primer selection for *fHbp* genotyping

The primers used for *fHbp* PCR and sequencing are listed in Table 2-5. The 'alternate' 1st round PCR primers were only used for extracts which had failed to yield a PCR product. On these occasions, the standard 2nd round PCR primers were used, however, *18692F* and *1871Ralt* were occasionally used as round two PCR primers if a primer mismatch was suspected.

2.6.2 Finalised *fHbp* PCR parameters

Table 2-6 lists the thermal cycler conditions that were identified as optimal following PCR primer optimisation. The standard primers round one and two primers sets required no additional MgCl₂ in the mastermix (concentration 1.5 mM).

Table 2-5: Oligonucleotide primers used for genotyping of *fHbp*

Identifier	Use	Direction	Sequence (5'-3')*	Ref
1869-2F	Standard 1 st round PCR	Forward	GAAGAAATCGTCGAAGGCATCAAAAC	(Lucidame et al., 2011a)
1871Ralt	Standard 1 st round PCR	Reverse	GACGGTTAAATCAGCTTG	
<i>fHbp</i> Rd2F	Standard 2 nd round PCR	Forward	GTATGCCCAAGGGCGAATTGAACC	n/a
<i>fHbp</i> Rd2R	Standard 2 nd round PCR	Reverse	GTGCGGATTTCCGGCAG(A/G)ATCA	
<i>fHbp</i> outerF	Alternate 1 st round PCR	Forward	TCGACCGCATCAAAAGAAATCCACC	
<i>fHbp</i> outerR	Alternate 1 st round PCR	Reverse	CCGAC(C/T)TGATAA(C/T)CGCTCAAAACGG	
GNA1870F	Outer sequencing primer	Forward	TGACCTGCCTCATTTGATGC	(Jacobsson et al. 2006)
<i>fHbp</i> seqR2	Outer sequencing primer	Reverse	AGGACGGG(G/A)CGGTT(G/A)AAATC	n/a
GNA1870S2	Internal sequencing primer	Forward	CAATCGAAGTGGACGGGCAG	(Jacobsson et al. 2006)
GNA1870S3	Internal sequencing primer	Reverse	TGTCGATTTGCCGTTTCCCTG	(Jacobsson et al. 2006)

Four of the PCR primers and one of the sequencing primers were designed for this study. The first round PCR primers as well as three of the sequencing primers were previously published.

Table 2-6: Thermal cycler parameters used during *fHbp* PCR analysis.

Primer set	PCR stage	Temperature (°C)	Duration	No of cycles
<i>fHbp</i> outerF and <i>fHbp</i> outerR	Taq Activation	95	15mins	X1
	Denaturation	95	30 secs	X35-45
	Annealing	63	30 secs	
	Elongation	72	130 secs	
	Final Extension	72	7 mins	X1
	Holding	4	∞	X1
1869-2F and 1871Ralt	Taq Activation	95	15mins	X1
	Denaturation	95	30 secs	X35-45
	Annealing	63	30 secs	
	Elongation	72	90 secs	
	Final Extension	72	7 mins	X1
	Holding	4	∞	X1
<i>fHbp</i> Rd2F and <i>fHbp</i> Rd2R	Taq Activation	95	15mins	X1
	Denaturation	95	30 secs	X30
	Annealing	63	30 secs	
	Elongation	72	90 secs	
	Final Extension	72	7 mins	X1
	Holding	4	∞	X1

The PCR cycle number varied from 35 to 45 depending on the Ct values of the specimens being tested, however, in most cases, 45 was used to maximise sensitivity.

2.6.3 Sequencing master mix and reaction

Sequencing primers were manufactured, prepared and stored as outlined in 2.4.1. Sanger sequencing reactions were performed using BigDye terminator v3.1 cycle sequencing kits (ThermoFisher, US). One reaction was prepared for each primer (Table 2-7).

Table 2-7: Sequencing master mix reagent volumes for *fHbp* genotyping

Reagent	Volume per reaction (μL)
BigDye® Terminator v3.1 Ready Reaction Mix	0.5
BigDye® sequencing buffer	1.75
Sequencing primer (5 μM)	0.66
Distilled water	6.09

For each sample to be sequenced, 9 μL of each primer mix was added to 1 μL of each PCR product. The plate was then placed on a thermal cycler and the sequencing reaction was performed using the conditions described in Table 2-8.

Table 2-8: Thermal cycler parameters used during *fHbp* sequencing reactions

Stage	Temperature (°C)	Duration	Number of cycles
Taq Activation	95	1 minute	x1
Denaturation	95	10 seconds	X25
Annealing	55	5 seconds	
Extension	60	4 minutes	
Holding	4	∞	x1

2.6.4 Clean-up of sequencing products

Sequencing reaction products were cleaned by ethanol precipitation. Sodium-Acetate buffer (3 M, Sigma Aldrich, UK) was added to 190 proof ethanol (Sigma Aldrich, UK) at a concentration of 4%. The solution was left to chill for 10 minutes before 40 μL was added to each sequencing reaction well. The plate was gently vortexed and incubated at room temperature for 10 minutes. The plate was then centrifuged at 2000 x g in a plate centrifuge for 20 minutes. Following centrifugation, the plate was gently inverted

on to absorbent paper roll to remove the supernatant. The plate was then placed in the centrifuge upside down (wells facing up) on a small piece of paper roll and centrifuged at 150 x g for 1 minute to remove residual supernatant. Following centrifugation, 100 µL of 70% ethanol was added to each pellet and the plate was once again sealed and centrifuged at 2000 x g for 20 minutes. The plate was again inverted and centrifuged upside down at 150 x g for 1 minute to remove the supernatant. The pellets were resuspended in 15 µL of HiDi formamide (ThermoFisher, US). The plate was then heated on a thermal cycler at 50 °C for 5 minutes.

2.6.5 Electrophoretic analysis of sequencing products

Capillary electrophoresis of sequencing products was performed using a 50 cm capillary on an ABI 3130xl genetic analyser using POP-7™ Polymer separation matrix (ThermoFisher, US). The plates were loaded on to an available platform and all appropriate reservoirs were filled with 1x Running Buffer (ThermoFisher, US) and nuclease-free water (Sigma Aldrich, UK). Using the ABI 3130xl software, appropriate sequence IDs were imported from a Microsoft Excel worksheet and the appropriate run parameters selected. Once all checks were complete, the electrophoresis run was performed. Basecalling was carried out automatically by the ABI 3130xl software. Electropherogram traces were saved in AB1 format.

2.6.6 *fHbp* sequence analysis

Analysis of *fHbp* sequence traces was carried out using Sequencher v4.7 (Gene Codes Corporation, Ann Arbor, MI USA). For each sample, the AB1 electropherogram files for all four sequencing primers were imported into Sequencher. A contig was assembled using the 'Assemble automatically' option utilising default assembly parameters. The resultant contig was then inspected and oriented in correct direction (5' to 3'). The start of *fHbp* (invariably beginning TGCAGC) was located and all upstream sequence was deleted from the contig. Poor quality traces found at the end of sequence traces were removed for each primer. The traces were realigned as required. The stop codon of the gene was then located and all downstream sequence was deleted from the contig. Any conflicting/ambiguous base calls were resolved by visually referring to the electropherogram. The *fHbp* allele and translated peptide IDs of the sequence were

determined using the PubMLST fHbp webpage's (pubmlst.org/neisseria/fhbp) single sequence query tool. Any unassigned alleles or translated peptide sequences were subsequently submitted to the PubMLST fHbp curator for numeric ID assignment. All *fHbp* sequencing results were stored in a Microsoft Access database.

2.7 *fHbp* genotyping data analysis

2.7.1 Clinical specimen, isolate and patient information

Anonymised information on PCR-positive clinical specimens (e.g. specimen type, genogroup, *ctrA*-specific and group-specific Ct values and any corresponding isolates) was provided by PHE MRU. Specimens from IMD cases with corresponding isolates or multiple non-culture specimens were identified and lists of testing were produced. For the comparative culture vs. non-culture analyses, the patient age in months and date of receipt of specimen/isolate for all IMD cases confirmed from January 2011 to March 2015 was provided by the PHE MRU.

To assess the distribution of fHbp variants amongst the prevalent CCs, fHbp and MLST genotyping data from isolates received over six epidemiological years were compiled. Genotypic data from isolates received from 2010/11 to 2014/15 (n=2306) were downloaded from the MRF MGL. To these, the corresponding data from isolates received in 2007/08 (n=613), produced using PCR sequencing as part of a previous study, were added (n=2919). The ST-269 complex was divided into two clusters, the ST-269 cluster or the ST-275 cluster based on the similarity of the MLST allelic profile (i.e. 4 or more identical alleles) to either ST-269 or ST-275, respectively.

2.7.2 Analysis of *fHbp* genotyping data

Analysis of raw data and preparation of graphs were performed using Excel 2013 (Microsoft, US). Fisher's exact tests and Cochran Mantel Haenszel tests were performed using Minitab 17 (Minitab Inc. US). Differences generating a p-value of <0.05 were considered statistically significant.

2.8 Selection of non-culture specimens for SureSelect XT assessment

2.8.1 Selection of non-culture specimens

For assessment of the SureSelectXT system, ten clinical specimens were selected from group B IMD cases that had also yielded viable isolates. In order to select samples with DNA from diverse strains, MLST data from all isolates (January 2011-July 2015) with a corresponding PCR-positive clinical specimen were downloaded from the PubMLST database (<https://www.pubmlst.org/neisseria>). Data on *ctrA*-specific Ct values, genogroup and specimen type on the corresponding PCR-positive specimens were provided by the PHE MRU. Five blood samples and five CSF samples were selected from cases caused by relatively diverse group B strains. A wide range of Ct values were selected in order to assess the sensitivity of the assay.

2.8.2 Estimation of DNA concentration within clinical specimens

To allow a practical assessment of the sensitivity of the SureSelectXT enrichment protocol, the DNA concentrations within the selected clinical specimens were estimated based on the *ctrA*-specific Ct values derived using the Taqman® PCR assay. To perform these estimations, *ctrA*-specific Ct values obtained from a dilution series of quantified meningococcal DNA extracts (Table 3-3) were plotted on a scatter graph in Microsoft Excel (Ct value against DNA concentration). An exponential trendline was applied to the data and the trendline equation was used to predict the DNA concentration from the Ct value of the selected clinical specimens.

The equation was also used to estimate the meningococcal DNA concentrations of hypothetical extracts producing all Ct values from 19 to 40. The Ct values of all PCR positive extracts (January 2016-December 2016) were compiled (data provided by PHE MRU) and used to estimate the proportion of extracts on which whole genome sequencing could be performed. To estimate the proportion of non-culture IMD cases that could be characterised, any duplicate specimens (i.e. additional samples from an individual patient) were removed until only one sample was included per case. The sample with the lowest Ct value (highest DNA concentration) was selected in each case.

2.9 SureSelect XT bait design, target enrichment and sequencing

Declaration: The methods described in the following section were performed solely by collaborators at University College London (UCL).

2.9.1 RNA bait design

Meningococcal-specific RNA baits were designed by collaborators at UCL using a previously-described Perl script (Depledge et al., 2011). Genomic sequences from complete meningococcal genomes on the NCBI GenBank database (n=77) and whole/draft meningococcal genomes within PubMLST (n=2898) were used as a template.

The bait library consisted of overlapping sequences of 120bp providing 2X coverage across all reference sequences. Sequences with specificity to the human genome (Hg19, accession number: GCF_000001405.13) were removed. Additional sequences were generated separately to match any gaps identified when aligned to complete meningococcal genomes. All bait sequences were uploaded to SureDesign (Agilent Technologies, US) and the oligonucleotides were manufactured by Agilent Technologies.

2.9.2 DNA extraction and enrichment of DNA libraries

DNA extraction, target enrichment, genome sequencing and *de-novo* contig assembly was performed by collaborators at UCL.

DNA extraction was performed using the EZ1 DNA Blood Kit on the EZ1 Advanced XL system (Qiagen, UK) following the manufacturer's instructions with an additional bead-beating bacterial cell lysis step. A total sample volume of 300 µL was used for each extraction. For samples with <300 µL available, dilution was performed using molecular grade water as required.

Target enrichment and DNA library preparation was performed in accordance with the SureSelectXT Target Enrichment System for Illumina Paired-End Multiplexed Sequencing Library protocol (Agilent Technologies, US). Sequencing of captured DNA libraries was performed on an Illumina MiSeq using the 500-cycle V2 kit (Illumina, US) in accordance with the manufacturer's protocol.

Read sequences were aligned to a human reference genome (Hg19, accession number: GCF_000001405.13) using BMap (version 37.00). Reads that did not align were corrected for

predicted sequencing error using Lighter (Song et al., 2014) and overlapping read pairs were merged using FLASH (Magoč and Salzberg, 2011). All merged paired reads and unpaired reads were then *de novo* assembled using SPAdes (Bankevich et al., 2012) without read error correction, assembler only, using k-mer values of 21, 33, 55, 77, 99 and 127.

2.10 Analysis of specimen-derived genomic data

2.10.1 Estimation of genome coverage and median read depth

Genome coverage and median read depth of each specimen-derived genome were calculated by collaborators at UCL by aligning screened reads against a pseudo-reference meningococcal genome. To generate the pseudo-reference, the complete genomes in the PubMLST database (n=237) were screened for their similarity to each sample by estimating distances based on shared K-mers using Mash (Ondov et al., 2016).

Pseudo-references were produced by aligning *de-novo* assembled contigs for each specimen against the corresponding complete genome using the BWA-MEM algorithm (Li, 2013). The resulting consensus sequences were used as pseudo-references to assess genome coverage and median read depth.

2.10.2 Comparison of isolate and specimen-derived genomes

In order to assess the quality of the specimen-derived genomes and to compare them with the corresponding isolate genomes, data on key genomic parameters were downloaded from PubMLST alongside typing data commonly used by the PHE MRU and other reference laboratories. The numbers of contigs, cumulative contig length, number of tagged loci and number of tagged NEIS loci were attained alongside the allele IDs for PorA VR1, PorA VR2, PorB VR, FetA VR, fHbp peptide, NHBA peptide, NadA peptide and MLST loci (abcZ, adk, aroE, fumC, gdh, pdhC, pgm). The ST and CC were also downloaded. Comparisons of the paired genomes were performed visually. Where data were missing, BLAST searches of the gene were carried out in the relevant genome in an attempt to identify the complete sequence on multiple contigs. Novel alleles were submitted for allelic ID assignment.

More comprehensive comparisons were then performed using the PubMLST Genome Comparator tool (Jolley and Maiden, 2010). In turn, each genome pair was selected and compared at all indexed NEIS loci (n=2652) using default parameters. NEIS loci that were present and complete in both genomes were compared visually for discrepant allele IDs. In each case, the discrepant alleles were downloaded and aligned using BioEdit v7. The number of nucleotide differences was recorded and if multiple differences were found and/or the gene was discrepant in more than one genome pair, the gene was BLAST searched using the NCBI Nucleotide BLAST tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to identify evidence of paralogy or regions of similarity among all *N. meningitidis* genomes within the NCBI Genbank database.

2.11 MEASURE assay strains, stocks and reagents

2.11.1 Strains used for assessment of MEASURE assay

Table 2-9 lists the strains used for assessment of the MEASURE assay. Twelve diverse strains were selected to assess paraformaldehyde (PFA) killing effectiveness and up to nine strains were used in order to compare MFI results between laboratories and agar types.

Table 2-9: Strains used for assessment of MEASURE assay.

Assessment	Isolate ID	fHbp peptide	Group	ST	Clonal Complex
PFA killing assessment	M01 240007	15	B	269	ST-269 complex
	M01 240070	16	W	184	ST-22 complex
	M01 240601	15	B	1791	ST-269 complex
	M04 240731	123	B	340	ST-41/44 complex
	M04 241215	4	B	41	ST-41/44 complex
	M05 240300	45	B	7299	ST-213 complex
	M14 240606	1	B	290	ST-32 complex
	M14 240616	13	C	11	ST-11 complex
	M15 240098	25	Y	23	ST-23 complex
	M15 240133	13	B	60	ST-60 complex
	M15 240465	22	W	11	ST-11 complex
	NZ98/254	14	B	42	ST-41/44 complex
Inter-laboratory and agar type comparisons	PMB 1135	55	B	44	ST-41/44 complex
	PMB 1745	45	B	2100	ST-213 complex
	PMB 2058	24	B	2976	ST-269 complex
	PMB 2802	19	B	43	ST-41/44 complex
	PMB 3242	4	B	4489	ST-41/44 complex
	PMB 3453	16	B	35	ST-35 complex
	PMB 3536	14	B	41	ST-41/44 complex
	M15 240821	47	B	1946	ST-461 complex
	M15 240859	13	B	275	ST-269 complex

2.11.2 Preparation of bacterial GC glycerol broth stocks

The MEASURE assay requires all strains to be pre-cultured within small aliquots of gonococcal (GC) broth with 14% (v/v) glycerol prior to plating on to GC agar during the MEASURE assay. To prepare the GC glycerol broth, glycerol (Sigma, UK) was added to GC broth (provided by Pfizer Vaccines), vigorously shaken and vortexed at high speed until a homogenous solution was achieved. For each strain, bacterial suspensions in GC glycerol broth were prepared at the required volume (determined by the number required) in a Universal tube (see 2.1.3). A volume of 50 µL was transferred to 1.5 mL screw-capped microtubes and stored at -80 °C.

2.11.3 Preparation of 1% (w/v) Bovine Serum Albumin in PBS

To prepare 1 L of 1% (w/v) Bovine Serum Albumin in PBS (BSA/PBS), 10 g of bovine serum albumin (BSA, Sigma Aldrich, UK) was added to 1 L of 1x PBS (without added Ca or Mg, Thermofisher, US). The solution was mixed until all BSA has dissolved before being filter sterilised using a Nalgene 0.2 µM filter unit (Thermofisher, US). BSA/PBS was stored at 4°C.

2.11.4 Preparation of 1% (v/v) Paraformaldehyde in PBS

Batches of 1% (v/v) PFA in PBS (PFA/PBS) were prepared by diluting 16% PFA (methanol-free, Alfa Aesar, US) in 1x PBS (without added Ca or Mg, Thermofisher, US). To prepare 480 mL of PFA/PBS, 30 mL of 16% paraformaldehyde was added to 450 mL of PBS. The solution was gently mixed and stored at 4°C.

2.11.5 Preparation of GCK broth for meningococcal cultivation

The GC broth, 4% Sodium Bicarbonate solution and Kelloggs supplement used to prepare GCK broth were provided by Pfizer Vaccines. GCK broth was prepared by supplementing GC broth with 4% Sodium Bicarbonate solution (1% v/v) and Kelloggs supplement (1% v/v). GCK broth was prepared prior to each assay run. Strains were cultured in 25 mL GCK broth within 250 mL Erlenmeyer flasks (Corning, US).

2.12 Assessing the killing effectiveness of PFA/PBS

2.12.1 “Dilution” method

For the initial killing assessments, a “dilution” method was used. Meningococcal strains were grown overnight (>16 hours) on CBA (refer to 2.1). For each strain tested, meningococcal cells were resuspended in two 15 mL Falcon tubes (Corning, US) containing 5 mL of GCK broth. The optical density (OD) of the suspensions were measured at 650 nm using a WPA S800 spectrophotometer (Biochrom Ltd., UK). Cells were added until an OD of >0.55 was achieved (greater than the OD used in the MEASURE assay). The suspensions were centrifuged for 4 minutes at 1825 x g and the supernatant was discarded. To one tube, 5 mL of PFA/PBS was added. As a control, 5

mL of GCK was added to the second tube. Both tubes were vortexed thoroughly and the incubated at 1-4 °C for the required time using a CoolRack (Biocision, US).

At the required time points, 2 µL of each suspension was transferred to microfuge tubes containing 1998 µL of GCK broth (1/1000 dilution). This was performed in order to reduce the effect of the fixative and to ensure sufficient growth after the dilution in the absence of the fixative (control). Then, 100 µL of each diluted suspension was plated onto a CBA plate, which was incubated at 37 °C with 5% CO₂ for ≥48 hours. The plates were then inspected for meningococcal growth and any colonies were counted (or recorded as confluent growth).

2.12.2 “Centrifugation” method

In order to increase the sensitivity and reliability of the later killing assessments, a new method was developed. Meningococcal strains were grown overnight (>16 hours) on CBA (refer to 2.1). For each strain tested and each time point required, meningococcal cells were resuspended in two 15 mL Falcon tubes (Corning, US) containing 3 mL of GCK broth. The OD of the suspensions were measured at 650 nm using a WPA S800 spectrophotometer (Biochrom Ltd., UK). Cells were added until an OD of >0.55 was achieved. The suspensions were centrifuged for 4 minutes at 1825 x g and the supernatant was discarded. To one tube, 3 mL of PFA/PBS was added. As a control, 3 mL of GCK was added to the second tube. Both tubes were vortexed thoroughly and the incubated at 1-4 °C for the required time using a CoolRack (Biocision, US). After the required time, the two tubes were centrifuged for 4 minutes at 1825 x g and the supernatant was discarded before 5 ml of GCK was added the tube. The tubes were vortexed to resuspend the cells and centrifuged once again for 4 minutes at 1825 x g. Finally, the supernatants were poured away and the pellets were resuspended in the residual broth remaining in the tube (200-300 µL) by agitating the bottom of the tube. This broth and the suspended cells were then transferred to CBA plates, which were incubated at 37 °C with 5% CO₂ for ≥48 hours. The plates were then inspected for meningococcal growth.

2.13 Finalised MEASURE assay protocol and data analysis

2.13.1 Overnight growth on GC agar

For each strain to be tested, 10 µL of the defrosted GC glycerol broth was plated for single colony isolation on GC agar. Agar plates were incubated overnight (16-18 hours) at 37 °C with 5% CO₂.

2.13.2 Liquid culturing of meningococci

All strains were grown within GCK broth to an OD of 0.5-0.55 (at 650 nm). To prepare initial meningococcal suspensions, 50-100 isolated meningococcal colonies were removed from the inoculated GC plates (following overnight incubation) and transferred to the Erlenmeyer flask containing 25 mL of GCK broth (2.11.5). The swabs were gently agitated along the inside of the vessel to remove the colonies and break up the cells. The OD of the suspensions was measured at 650 nm using a WPA S800 spectrophotometer (Biochrom Ltd., UK). Broth suspensions with ODs of 0.15-0.2 were transferred to a Minitron shaking incubator (Infors HT, Germany) and incubated at 37 °C with 150 rpm shaking. To suspensions with ODs of <0.15, more meningococcal colonies were added before being re-tested. To suspensions with ODs of >0.2, more GCK broth was added before being re-tested. This was repeated until all strains had achieved the starting OD of 0.15-0.2 and were incubated.

After one hour of incubation, the flask of each strain was removed from the incubator and the OD was again measured and recorded before the flask was returned to the incubator. The OD of the suspensions were then periodically measured and recorded as required until an OD of 0.5-0.55 was achieved. Upon reaching this OD, 5 mL of the suspension was transferred to a 15 mL Falcon tube (Corning, US) using a sterile pastette. The broth in the tube was then cooled to 1-4 °C in a CoolRack (Biocision, US). Suspensions that exceeded this OD were discarded and those strains were repeated on a subsequent assay run.

After 2-10 minutes, the Falcon tubes were centrifuged for 4 minutes at 1825 x g. Following centrifugation, the supernatant was carefully poured in to disinfectant. The tube was sealed and the bottom of the tube was agitated in order to disrupt the pellet

within the residual GCK broth. To the pellet, 5 mL of PFA/PBS was added and the tube was sealed and vortexed at high speed to resuspend the cells. This was repeated for all tubes before they were incubated at 4 °C for at least 16 hours.

2.13.3 Assay Day Three (cell staining and acquisition)

The fixed meningococcal cells were antibody stained using a three-step procedure. The reagents used to staining of the fixed meningococcal cells are described in Table 2-10.

Table 2-10: Staining reagents used in MEASURE assay.

Staining step	Antibody/reporter	Manufacturer	Working concentration
Primary	IgG control (non-specific) and MN86-994-11-1 (fHbp-specific)	Sponsor	6.7 µg/mL
Secondary	Biotinylated anti-mouse IgG (Goat)	Jackson Immuno-research. US (Cat: 115-065-164)	10 µg/mL
Tertiary	SA-PE (Streptavidin bound to Phycoerythrin)	BD Biosciences, Cat # 554061	5 µg/mL

Following at least 16 hours incubation at 4°C, the fixed cells were vortexed to resuspend the cells. For each strain, 50 µL was added to each of four microtitre wells as illustrated in Figure 2-1. Primary staining was carried out in parallel using both a non-specific isotype control and the fHbp-specific monoclonal (MN86-994-11-1). Staining was carried out in 96-well microtitre plates and each strain was stained in quadruplicate (Fig. 2-1). Two of the four quadruplicates were stained with a non-specific control primary antibody and the remaining two were stained with the fHbp-specific antibody (MN86-994-11).

Figure 2-1: Configuration of staining plate during primary staining in the MEASURE assay. Each strain was stained twice with the control primary antibody (mouse IgG) and twice with the fHbp-specific primary antibody (MN86-994-11-1).

Well	1	2	3	4	5	6	7	8	9	10	11	12		
mAb	mIgG	MN86-944-11-1	mIgG	MN86-944-11-1	mIgG	MN86-944-11-1	mIgG	MN86-944-11-1	mIgG	MN86-944-11-1	mIgG	MN86-944-11-1		
Duplicate	a	a	b	b	a	a	b	b	a	a	b	b		
A	Strain #1			Strain #2									Strain #3	
B	WASH WELLS													
C	Strain #4			Strain #5									Strain #6	
D	WASH WELLS													
E	Strain #7			Strain #8									Strain #9	
F	WASH WELLS													
G	Strain #10			Strain #11									Strain #12	
H	WASH WELLS													

mAb: monoclonal antibody

The microtitre plate was then centrifuged at 1825 x g for 4 minutes. The supernatant was removed by inverting the plate. The cells were washed by resuspending the cells pellets in 200 μ L of BSA/PBS. The plate was then centrifuged at 1825 x g for 4 minutes before the plate was inverted to remove the BSA/PBS.

The primary antibodies were prepared by diluting stock antibodies to the working dilution in BSA/PBS. 50 μ L of the appropriate antibody was added to each well as indicated in Figure 2-1. The cell pellets were then resuspended in the antibody solutions. The plate was incubated at 4 °C for 30 minutes.

Following incubation, 150 μ L of BSA/PBS was added to each well before the plate was centrifuged at 1825 x g for 4 minutes. The supernatants were removed by plate inversion and the cells were re-suspended in 200 μ L of BSA/PBS. The plate was once again centrifuged at 1825 x g for 4 minutes and the supernatant discarded. The secondary antibodies were diluted to the working concentration in BSA/PBS, 50 μ L was added to each microtitre well and the cells were resuspended. The plate was incubated for 30 minutes at 4 °C.

The plate was then washed twice in BSA/PBS (as described following the primary antibody incubation) before the SA-PE was diluted to the working concentration in BSA/PBS and added to the cells (50 μ L). The plate was covered (to protect from light) and incubated for 30 minutes at 4 °C.

The plate was once again washed twice in BSA/PBS before the pelleted cells were resuspended in 200 μ L of PFA/PBS. Prior to analysis of the stained cells, a ¼ dilution of the cells was performed by transferring 50 μ L of each well to the corresponding well of a new microtitre plate which contain 150 μ L of PFA/PBS. To the wash wells (rows B, D, F and H), 200 μ L of BSA/PBS was added. The plate was sealed with a perforatable plate sealer.

For wells containing the stained cells, 20,000 events were captured (using pre-defined FSC-A/SSC-A gate) at a 'slow' flow rate (14 μ L/minute) using an Accuri C6 flow cytometer (Becton Dickinson, US). Acquisition was performed beginning at well A1 and

working down each column in turn (Fig. 2-1). The intervening wash wells were acquired for 5 seconds in order to reduce carryover.

Following acquisition, the mean MFI within the FL-2 channel for each stained wells were recorded. As such, two fHbp-specific MFI values were recorded for each strain in each assay run.

2.13.4 Analysis of MEASURE results data

For each strain tested, median values and inter-quartile ranges were calculated using Microsoft Excel. Using Minitab 17 (Minitab Inc., US), Mann-Whitney tests were performed in order to compare differences in MFI. Differences generating a p-value of <0.05 were considered statistically significant.

3.0 RESULTS

3.1 The development of a non-culture *fHbp* genotyping assay

Declaration: Much of the work described in the following section was published in Clark et al. 2014 and parts of the following paragraphs have been amended from this publication. This article was written solely by me and all words used are my own.

3.1.1 Primer selection and optimisation

3.1.1.1 Existing primers

Primers *1869-2F* and *1871Ralt* were pre-optimised and have been used in previous studies (Lucidarme et al., 2011a). The complementary binding sites were checked against the conservation panel (Appendix I) to assess level of conservation. *1869-2F* appeared much conserved, with only five isolates showing a single base-pair mismatch within the binding site. None of these were at the 3' end and so are unlikely to prevent amplification. For *1871Ralt*, however, 171/391 (43.7%) of isolates featured single base pair mismatches in the 5' portion (first 5bp) of the binding site. These were deemed sufficiently conserved and, due to their out-lying positions, were selected for use in PCR round-one. These primers generate a product of between 1649 and 1876 bp. Three of the four sequencing primers selected for the assay were also previously published (Jacobsson et al., 2006). The fourth primer of this set, *gna1870R*, exhibited secondary binding to an alternate site within *fHbp* resulting in poor quality sequence traces. An alternative reverse sequencing primer was, therefore, required as well as 'nested' round-two PCR primers.

3.1.1.2 Design of new *fHbp* primers

To facilitate the use of a nested PCR protocol and to offer flexibility in the use of the existing primers, two PCR primer pairs, *fHbpouterF* & *fHbpouterR*, and *fHbpRd2F* & *fHbpRd2R*, were designed. Moreover, a new sequencing primer, *fHbpseqR2*, was designed to replace *gna1870R* as the outermost reverse sequencing primer.

Firstly, a search for candidate PCR primer sites outside of/flanking the selected round-one primers was made using sequences from 15 complete genomes (Appendix I). Seven 'outer' forward candidates and five reverse candidates were identified. Only

three forward candidates and one reverse candidate showed sufficient sequence conservation among 391 English and Welsh meningococcal isolate genomes within the PubMLST database (University of Oxford, <http://pubmlst.org/neisseria/>). Of the three forward candidates, two exhibited binding to sites within the genes and intergenic regions flanking *fHbp*. The remaining forward candidate, named *fHbpouterF7*, was sufficiently specific and suitable for use with the remaining reverse candidate *fhbpouterR5a* (i.e. no dimers, similar melting temperatures). These were selected as the 'outer', alternate primer set and, for clarity, were renamed *fHbpouterF* and *fhbpouterR*, respectively. These primers yield a PCR product of approximately 2200bp (depending on the presence of internal indels).

To identify candidates for the standard round-two PCR primers, sequences proximate to the round-one primer sites were used. For the forward site, five suitable candidates were initially identified but only one exhibited sufficient conservation among PubMLST genomes (*fhbpRd2F2*). To facilitate primer extension, this candidate was modified to include a 3' terminal dinucleotide CC. In order to retain its original GC%, a CC dinucleotide was removed from the 5' end. This primer was renamed *fhbpRd2F2a* to reflect these changes.

The PrimerQuest software generated only two suitable reverse candidates among the corresponding search region. Both primers showed minimal secondary binding within *fHbp* and in the flanking sequences, however, only one (*fHbpRd2R2*) exhibited sufficient conservation for routine use. This primer had to be modified through the removal of the three 3' end bases and the addition of a guanine at the 5' end in order to remove variable bases and increase the melting temperature, respectively. The modified primer was named *fHbpRd2R2a*. *fHbpRd2R2a* showed acceptable conservation among the 391 genomes analysed except for position 18 (5' to 3'), at which the majority of isolates (234/391, 59.8%) had a single mismatch (a cytosine in place of the original thymine). Due to this dichotomic nucleotide residue among invasive isolates, a degenerate base was introduced at this position. These two primers were selected for use as standard round two PCR primers and yield a PCR product of approximately 1500bp. They were renamed *fHbpRd2F* and *fHbpRd2R*. The complementary binding sites of all four newly-designed PCR primers are located within

genes flanking *fHbp*, NMB1869 (in MC58) coding for fructose-bisphosphate aldolase upstream of *fHbp* and NMB1871 coding for a putative peptidase downstream of *fHbp*.

The search for a new reverse sequencing primer yielded three candidates. All three exhibited a good level of conservation and showed no significant non-specific binding to the second round PCR product. The three candidates were tested by sequencing using standard sequencing protocol on PCR products from the seven sensitivity panel isolates (Appendix II). All candidates produced acceptable reverse sequence traces, however, the traces produced by *fHbpseqR2* appeared to feature less background noise than the other two primers. This primer was then tested on products from non-culture samples. Once again the traces were of sufficient length and clarity. This primer was chosen as the standard outer reverse sequencing primer. It contains two degenerate sites at positions 9 and 15. These were introduced to improve the homology of the primers among isolates of the conservation panel (Appendix I).

3.1.1.3 Optimisation of newly-designed PCR primers

Initial optimisation of the second round PCR primers (*fhbpRd2F* and *fHbpRd2R*) involved testing three different MgCl₂ concentrations (1.5 mM, 3 mM and 4.5 mM) and five different annealing temperatures (51 °C, 55 °C, 58 °C, 60 °C and 63 °C) over two PCR runs. Four of the seven isolates listed in Appendix II were used (highlighted by *). The resulting products are shown in Figure 3.1.

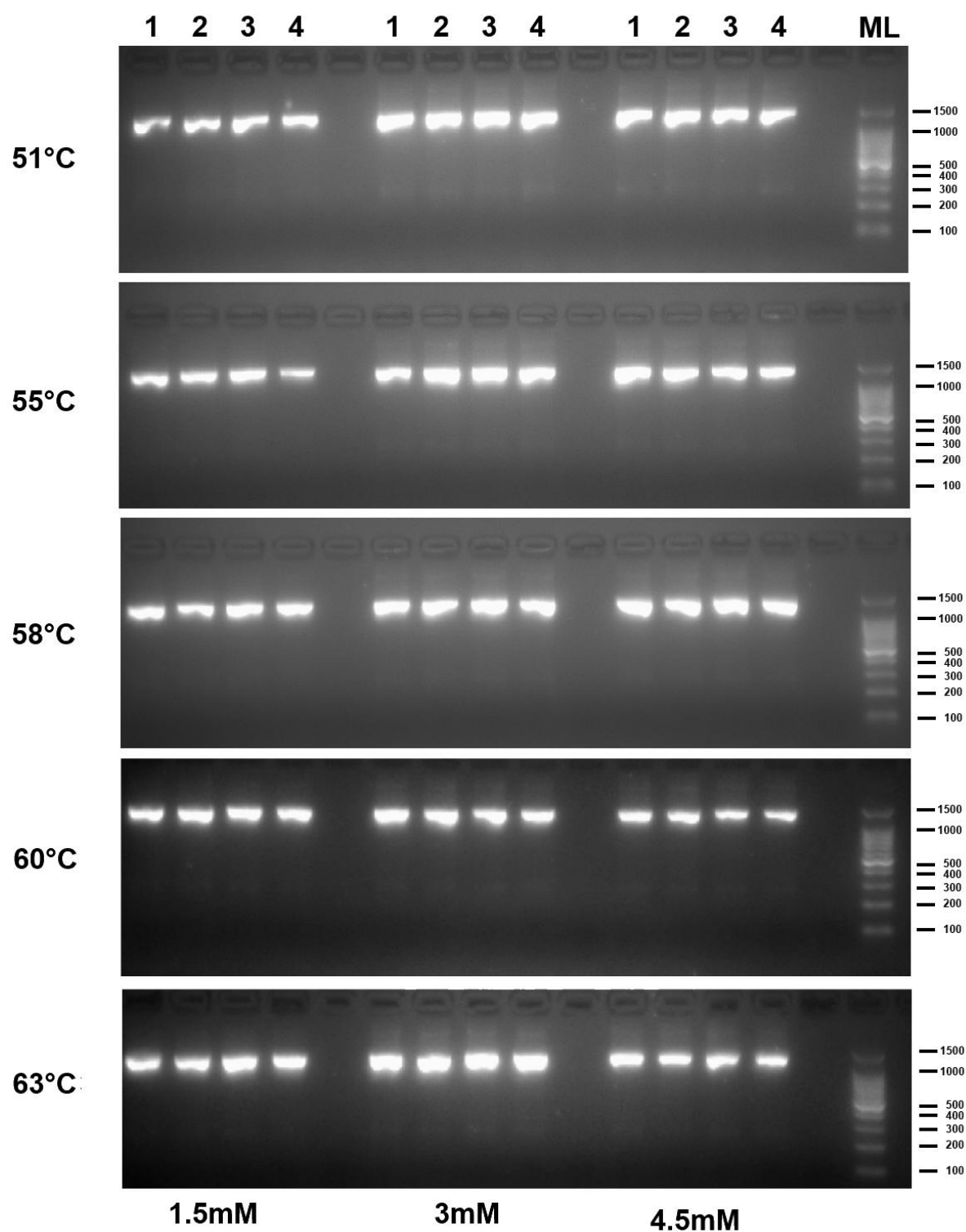


Figure 3-1: PCR products from four isolates after 35 cycles using *fhbpRd2F* and *fHbpRd2R*. The numbers above indicate the isolate number as described in Appendix II. The MgCl₂ concentration used is indicated at the bottom of the image. The corresponding annealing temperature used is indicated to the left of each row. ML=molecular ladder. Ladder size (bp) is indicated to the right of each panel.

Strong, defined bands of approximately 1500 bp can be seen for all conditions tested. Very faint non-specific products of 200-300 bp can also be seen in almost all conditions. Based on these findings, a wide range of annealing temperatures could be used; however, 63 °C was chosen as the optimum temperature to reduce the likelihood of non-specific binding. No additional MgCl₂ was deemed required (final selected concentration: 1.5mM).

The final parameter to be optimised was the cycle number. It was predicted that restricting the cycle number for the second PCR round to a minimum would help to reduce non-specific amplifications, which may be more likely to occur when testing non-culture extracts containing host DNA. Using the complete seven-isolate panel (Appendix II), the round-two primers were tested using the finalized conditions but at 25 and 30 cycles (previously tested at 35). Whilst the bands after 25 cycles are moderately bright, after 30 cycles, very strong PCR bands, similar to those observed after 35 cycles were seen (Fig. 3-2). It was, therefore, decided to restrict the second round to 30 cycles in order to reduce the potential for non-specific product generation.

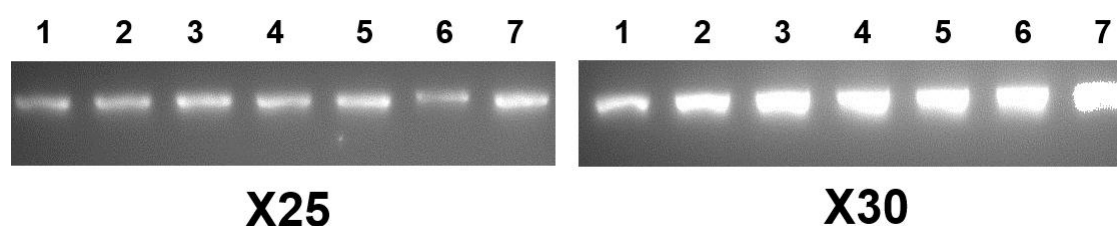


Figure 3-2: PCR products using round-two *fHbp* primers on 2% agarose gel following optimisation of PCR cycle number. The numbers above the images correspond to the order in which the isolates are listed in Appendix II. The PCR cycle numbers used are indicated below the images.

In order to optimise the alternate outer primers, the four isolates used previously for optimisation (Appendix II) were tested in a single PCR round (2 µL template in 25 µL reaction) using three annealing temperatures (58 °C, 60 °C and 63 °C) and three MgCl₂ concentrations (1.5 mM, 3 mM and 4.5 mM). As with the second round primers, the annealing temperature did not appear to influence the product yield to a great degree (Fig. 3-3). The MgCl₂ concentration, however, did impact on the result with the yield at 4.5 mM noticeably lower and with non-specific bands present.

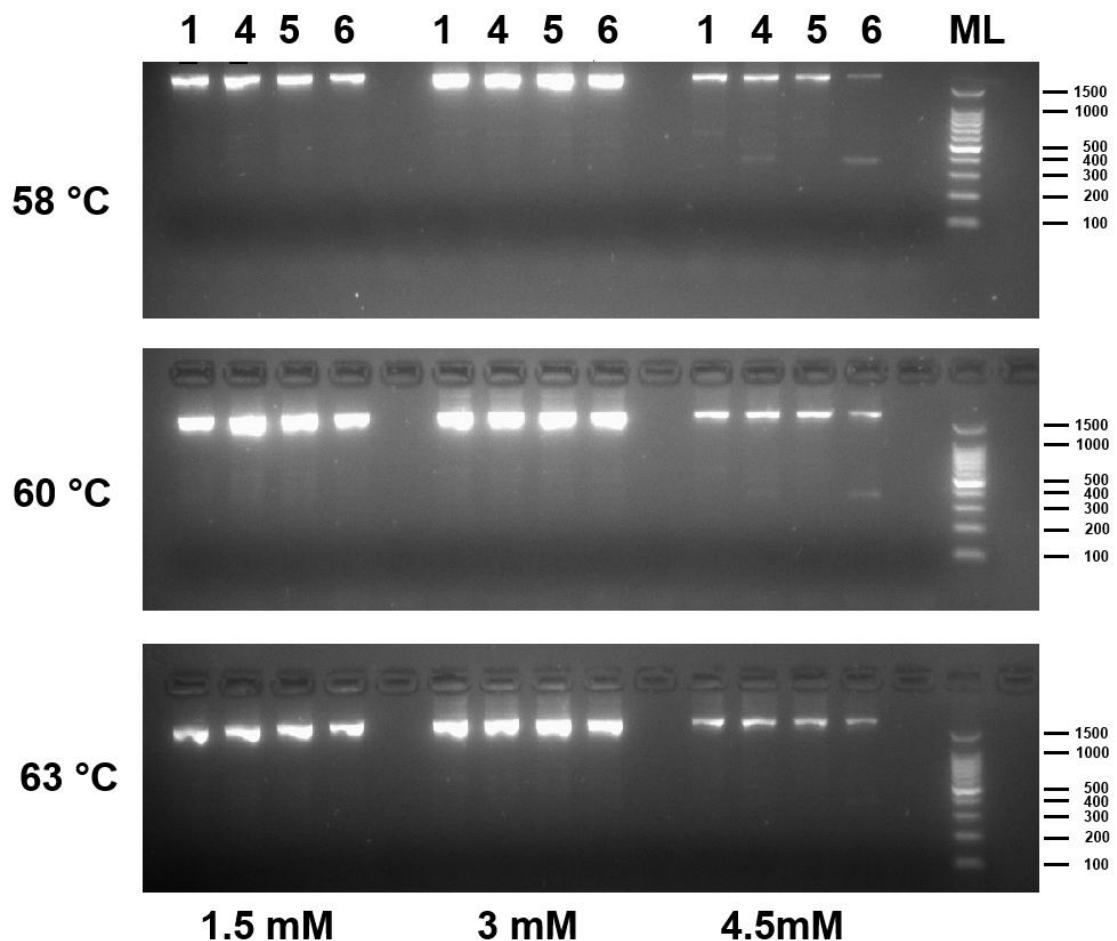


Figure 3-3: PCR products following a single PCR round (35 cycles) with *fHbpouterF* and *fHbpouterR* using extracts from four isolates. The isolate numbers above the image correspond to those in Appendix II. Annealing temperatures are indicated to the left of each panel and MgCl₂ concentrations are shown below. ML=molecular ladder. Ladder size (bp) is indicated to the right of each panel.

At the highest annealing temperature, the 3mM MgCl₂ concentration appeared yield a greater amount of product and relatively lower amounts of non-specific amplification. As these will be used as round one PCR primers, a cycle number of 45 should be used in order to maximise the sensitivity of the assay. The finalised PCR conditions for the *fHbp* PCR primers are listed in Table 2-6.

3.1.2 Assessment of analytical sensitivity

A dilution series of DNA extracts from seven diverse isolates (Appendix II) were used to assess the analytical sensitivity of the PCR protocol to the target template. Table 3-1 contains the estimated DNA concentration of each extract dilution as well as the calculated number of meningococcal genomes per microlitre.

Table 3-1: Estimated DNA concentration and number of meningococcal genomes per microliter for each extract dilution of the analytical sensitivity panel.

Extract dilution	Estimated DNA concentration	Number of meningococcal genome copies (per μL)
Original adjusted extract	6ng/ μL	2.53×10^6
10^{-1}	600pg/ μL	2.53×10^5
10^{-2}	60pg/ μL	2.53×10^4
10^{-3}	6pg/ μL	2530
10^{-4}	600fg/ μL	253
10^{-5}	60fg/ μL	25.3
10^{-6}	6fg/ μL	2.53
10^{-7}	600ag/ μL	0.253
10^{-8}	60ag/ μL	0.0253

PCR amplification using round-one primers (non-nested) yielded strong visible PCR products for all seven isolate extracts down to the 10^{-4} dilution (Fig 3-4). Faint bands were visible for isolates down to the 10^{-5} dilution. This indicates a PCR round one analytical sensitivity limit of 60 fg/ μL or 25.3 genome copies/ μL . As 5 μL of initial template was used for round one, this suggests at least ~127 copies are required in a round one reaction mix for visualisation of PCR product.

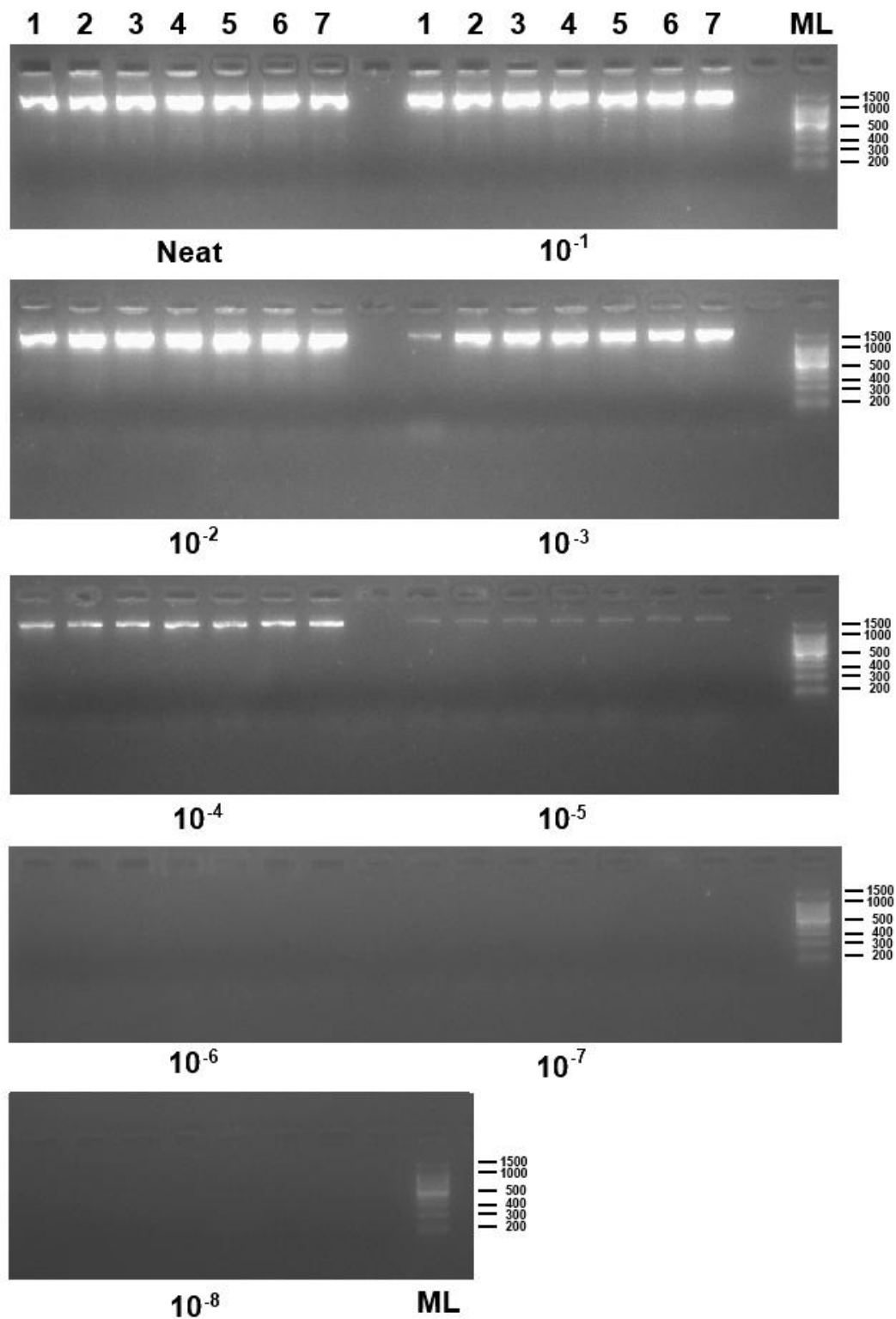


Figure 3-4: PCR products following fHbp PCR round one reaction using diluted extracts from the seven isolates of the analytical sensitivity panel. A volume of 5 μ L of extract was added to a 25 μ L reaction. The numbers above the image correspond to the isolate number in Appendix II. The extract dilution is indicated below each set of wells. ML=molecular ladder. Ladder size (bp) is indicated to the right of each panel.

PCR round one reaction mix from those extracts that produced faint or no visible bands (10^{-5} , 10^{-6} , 10^{-7} and 10^{-8}) were transferred to PCR round two reactions using 1 μL , 2 μL and 5 μL of template in 25 μL reactions. All isolates produced strong bands at 10^{-5} and 10^{-6} (Fig. 3-5). At 10^{-7} dilution, five of the seven isolates produced strong PCR products. No products were visible for any of the isolates at 10^{-8} . As some of the 10^{-7} extracts yielded a product, the analytical sensitivity of the nested PCR protocol was determined to be between 600 ag/ μL and 6 fg/ μL (between 1.2 and 12 genome copies per reaction).

The intensity of the bands increased with volume of round one mix used in round two reaction, however, the volume used did not appear to influence which of the isolate extracts amplified (i.e. negative extracts were negative at all transfer volumes tested). Larger transfer volumes also resulted in greater non-specific product amplification as evidenced by additional bands and smears on the gel. Based on these results, it was decided that 2 μL would be used as the standard transfer volume as it would provide a reasonable chance of product visualisation whilst curtailing spurious non-specific product amplification.

It was hypothesised that the volume of DNA extract used in the first round of the nested PCR may be influential in determining the likelihood of gaining a PCR product at the lower DNA concentrations (such as those in 10^{-7} and 10^{-8} dilutions). To test this hypothesis, differing volumes (5 μL , 10 μL and 20 μL) of the 10^{-7} and 10^{-8} extracts were added to the first round of nested PCR reactions. The results (Fig. 3-6) indicate that using greater extract volumes improve the chances of amplification. All of the 10^{-7} extracts yielded strong PCR products when 20 μL of extract was used, but only two and six of the seven 10^{-7} extracts successfully amplified when 5 μL and 10 μL was used, respectively. For the 10^{-8} extracts, four produced visible product, all were produced when using 20 μL of extract. These findings suggest that the analytical sensitivity could in some cases be increased down to 60 ag/ μL (~ 0.5 genome copies per reaction) if at least 20 μL of extract was used in the reaction.

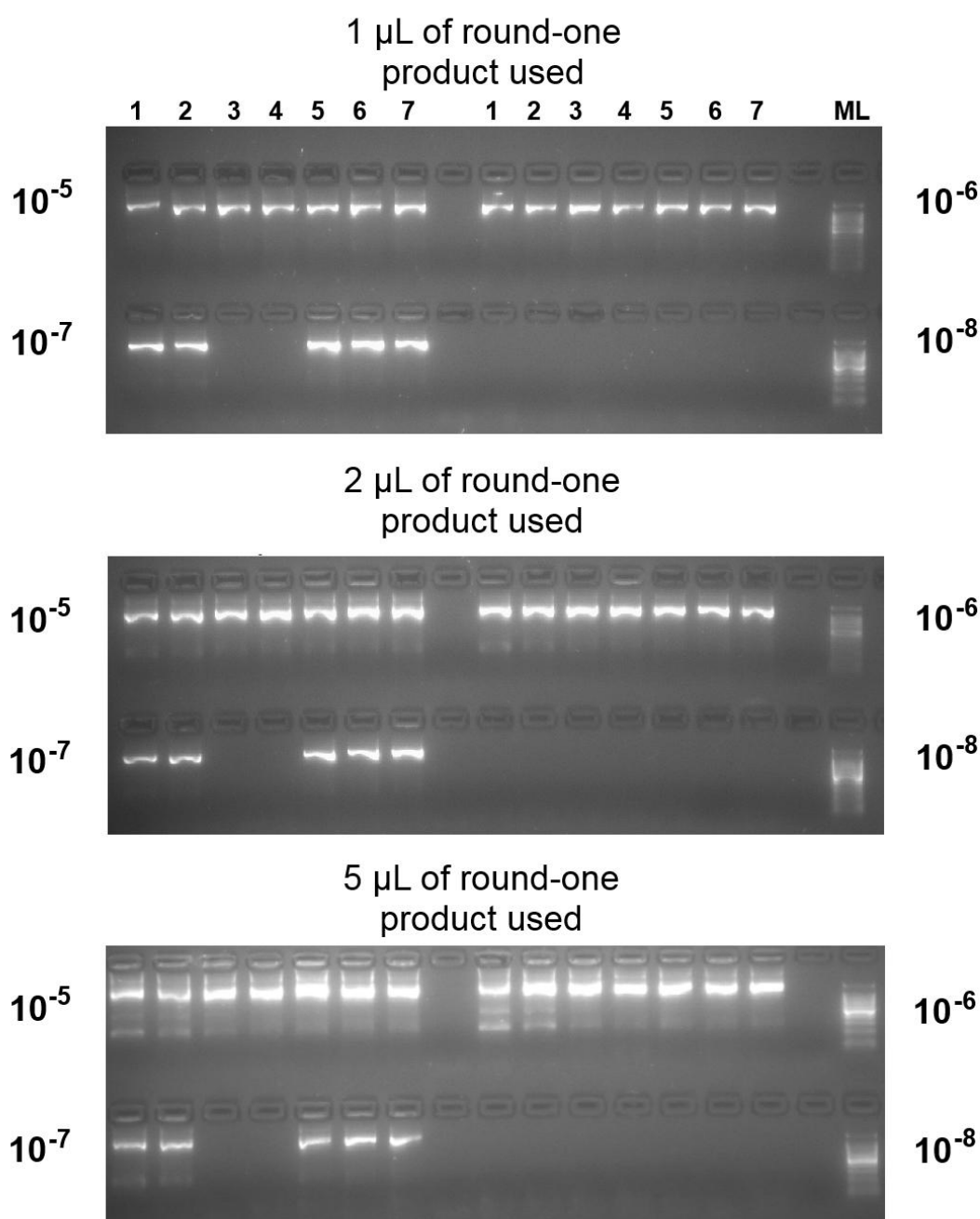


Figure 3-5: PCR products following 25 μ L *fHbp* PCR round two reactions using 1 μ L, 2 μ L and 5 μ L of PCR round one product for extract dilutions 10^{-5} , 10^{-6} , 10^{-7} and 10^{-8} as DNA template. The numbers above the top panel correspond to the isolate numbers in Appendix II. The volume of round-one product transferred to the second PCR round is indicated above each panel. The extract dilution is specified adjacent to each set. ML=molecular ladder: top band= 1500bp.

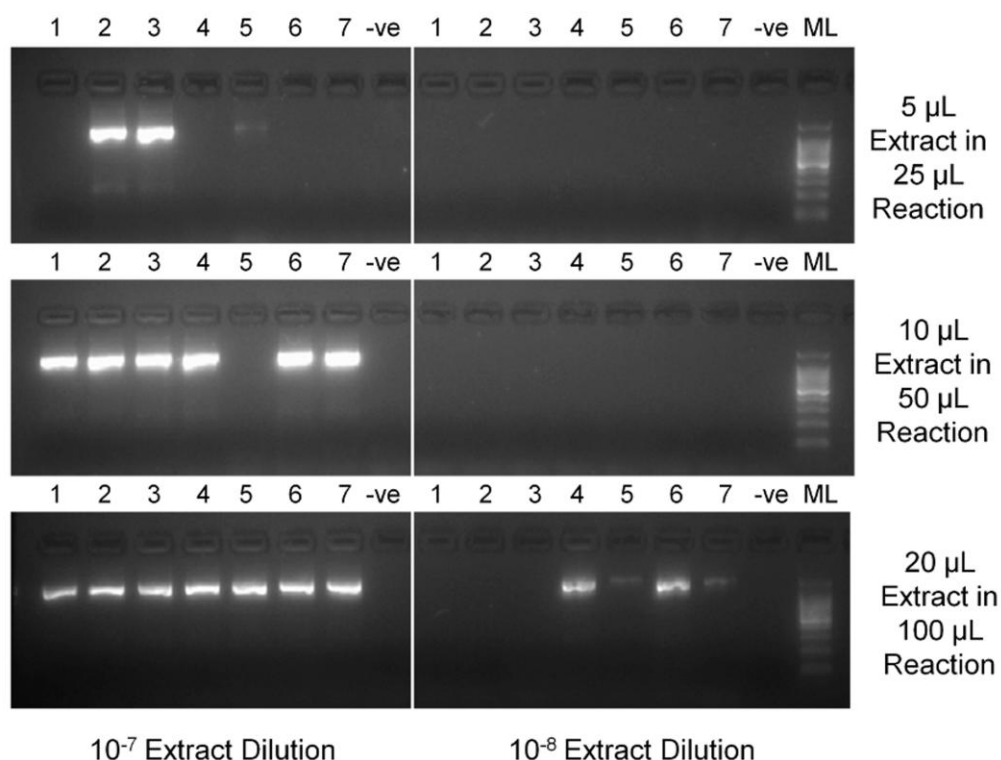


Figure 3-6: PCR products following nested *fHbp* PCR reactions using 10^{-7} and 10^{-8} DNA extracts. Three differing round one template/reaction volumes were tested. The template/reaction volumes are indicated to the right of each row. ML=molecular ladder: top band= 1500bp.

3.1.3 Correlative Taqman® analysis of analytical sensitivity extracts

Two different *ctrA* Taqman® assay formats: the original ‘wet mix’ assay introduced in 2003, and the more recent lyophilised assay (refer to 1.9) were used to determine the Ct values of the diluted extracts of the analytical sensitivity panel. The Ct values derived following analysis of the diluted extracts using the wet master mix assay are shown in Table 3-2. The Ct values increased as the DNA concentration decreased. The Ct values ranged from 16.8 to 44.7. The average increase in mean Ct value between each successive 10-fold dilution was 3.53. The final two dilutions for each isolate were tested in triplicate to compensate for possible stochastic sampling effects. Indeed, such effects were observed with one of the seven 10^{-7} extracts failing to produce a positive result in all three attempts. Of the 10^{-8} extracts, 3/7 failed to produce a positive result. None of the extracts at these two dilutions produced positive results in triplicate.

Table 3-2: *ctrA*- specific Ct values of diluted isolate DNA extracts using 'wet' Taqman® assay

Isolate	Ct Value per dilution											
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷ a	10 ⁻⁷ b	10 ⁻⁷ c	10 ⁻⁸ a	10 ⁻⁸ b	10 ⁻⁸ c
M08 240297	16.8	20.2	23.8	27.2	30.8	34.3	neg	40.8	38.6	neg	neg	neg
M07 240954	17	20.6	24.1	27.4	30.7	33.6	neg	neg	neg	44.7	neg	neg
M07 241036	20	23.4	26.9	30.3	33.4	36.9	neg	39.6	neg	neg	neg	neg
M07 241073	17	20.3	24	27.3	30.5	34.7	neg	neg	39.9	neg	neg	neg
M07 240725	17.1	20.5	24.1	27.3	30.5	34.1	37.5	37.5	neg	40	neg	42.2
M08 240113	17.2	20.8	24.3	27.6	30.7	34.9	39.6	37.4	neg	41.8	neg	neg
M08 240032	17	20.3	24	27.5	30.9	33.9	neg	39.4	36.6	43.7	neg	neg
Control	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
Ct Average	17.44	20.87	24.46	27.8	31.07	34.63	38.55	40.2	38.37	42.55	neg	42.2
								39.04			42.38	

neg= threshold not reached after 45 cycles

Table 3-3: *ctrA*- specific Ct values of diluted isolate DNA extracts using lyophilised Taqman® assay

Isolate	Ct Value per dilution											
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷ a	10 ⁻⁷ b	10 ⁻⁷ c	10 ⁻⁸ a	10 ⁻⁸ b	10 ⁻⁸ c
M08 240297	16.8	20.1	25	28.3	32.1	36.3	41	neg	neg	neg	neg	neg
M07 240954	17.4	20.7	25.3	28.9	32.3	38.7	neg	neg	neg	neg	neg	neg
M07 241036	20.6	25.8	29	33	38.1	41	neg	neg	neg	neg	neg	neg
M07 241073	17	20.8	25.4	28.7	32.9	38.5	neg	neg	neg	neg	neg	neg
M07 240725	17.4	22	25.2	neg	32.3	36.1	neg	neg	neg	neg	neg	neg
M08 240113	17.5	22.1	25.3	27.6	32.2	36.5	neg	neg	neg	neg	neg	neg
M08 240032	17.2	21.8	25.3	28.4	32	37.9	neg	neg	neg	neg	neg	neg
Control	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
Ct Average	17.7	21.85	25.79	29.15	33.13	37.86	41	neg	neg	neg	neg	neg
								41			neg	neg

neg= threshold not reached after 45 cycles

The results show that the older Taqman® assay using 'wet mix' is more sensitive than the lyophilised assay introduced in 2011. At the nested assay's analytical sensitivity limit (after one attempt, 10⁻⁶), a mean Ct value of 37.86 was obtained using the lyophilised assay which was used for the majority of the specimens tested.

The Ct values produced by testing the diluted extracts using the reconstituted lyophilised assay plate are shown in Table 3-3. The Ct values of 10^{-1} dilution extracts were very similar to those observed using the wet mix assay. Also, the results once again show a consistent increase in Ct value as the DNA concentration decreased; however, using the lyophilised assay, the average increase in mean Ct value at each successive dilution was higher than the wet mix assay at 4.03. This translates into a lower sensitivity, which in turn results in a lack of positive results for all of the 10^{-8} extracts and only one positive result for one of the 10^{-7} extracts. The Ct values between each isolate appeared to be reasonably similar with the exception of M07 241036, which appeared to be approximately three Ct values higher than the corresponding extracts at each dilution. The reasons for this were unclear, but are likely to be due to an error during dilution and/or quantification.

3.1.4 Specificity of the *fHbp* PCR assay

In order to assess the ability of the newly-developed assay to amplify *fHbp* from diverse meningococci, a panel of 96 isolates and corresponding non-culture samples (from the same patient) was compiled. eBURST and Splitstree analyses of the isolate MLST data were used to illustrate the diversity of the panel. The annotated eBURST and SplitsTree diagrams can be found in Appendix III. The isolates were selected from disparate STs and rMLST profiles, indicating genetic diversity.

The selected isolates and non-culture specimens, along with the corresponding MLST and *fHbp* data, are listed in Appendix IV. For the isolates, the two *fHbp* PCR rounds were carried out individually. Using the PCR round one primers, PCR products of appropriate size (~1750bp) were generated from all 96 isolate extracts. Using the PCR round two primers, however, one of the 96 extracts (M11 240189) failed to produce any discernible PCR product. Three subsequent attempts also failed to amplify an appropriate product. To assess the complementarity of the primers to the isolate genome, the *fHbp* flanking regions of M11 240189 were downloaded from PubMLST and aligned against the PCR round two primer sequences. Three base mismatches were identified in the 3' end of the *fHbpRd2R* primer binding site (Fig.3-7).

To assess the prevalence of these mismatches among English and Welsh invasive isolates, the sequences containing the *fhbpRd2R* binding regions amongst all MRF MGL isolate genomes from 2010/11 and 2011/12 (n=923) were downloaded and aligned. Only one other isolate (M11 240984) was found to feature this mismatch.

<i>fhbpRd2R</i>	TGATYCTGCCGGAATCCGCAC
(A)	GAAC CCTA
(B)	GAATG T C GCTG

Figure 3-7: Comparative alignment of the *fhbpRd2R* PCR primer (3' to 5') against (A) the majority of isolate genomes within the MRF MGL (921/923) and (B) a small number (2/923) of MRF MGL genomes as well as genomes from two of the six *N. lactamica* isolates tested. Single dots indicate bases matching the PCR primer.

Sequencing of the PCR products from the isolates was successful for all but one (M11 240402). The data downloaded from the corresponding genome indicates that M11 240402 is an *fhbp*-null isolate (Appendix IV) explaining lack of sequence traces. All of the 94 sequenced *fhbp* amplicons matched the alleles amongst the corresponding genomes (Appendix V).

The DNA extracted from the specimens was tested using the finalised nested PCR protocol. Of the 96 extracts tested, 83 (86.5%) produced appropriate PCR products on the first attempt (Run 1, Appendix V). Two subsequent runs (Runs 2 and 3) were performed on non-amplifying extracts using the finalised PCR conditions. These two runs yielded six additional PCR products. The remaining seven extracts that failed to generate a PCR product were tested in one final run using increased round one template/reaction volumes (20 µL/100 µL) and with increased PCR cycles (round one increased to 45, round two increased to 35). PCR products were obtained for two of the seven extracts. The remaining five extracts were considered PCR negative and no additional attempts at amplification were made. In total, 91 of the 96 clinical specimen extracts (94.8%) yielded an appropriately-sized PCR product after four attempts (Table 3-5). The clinical specimen corresponding to isolate M11 240189 (M11 909694) yielded

an acceptable PCR product despite, presumably, harbouring the same round two primer mismatch as the isolate.

The sequencing results for all amplified products are shown in Appendix V. All of the products from the clinical specimen extracts were successfully sequenced and all but two of the *fHbp* alleles matched those of the corresponding isolate. For the two discordant extracts (M11 922854 and M11 915746), both alleles differed from the corresponding allele by a single base. Re-amplification and sequencing of the extracts yielded *fHbp* alleles that matched the corresponding isolates suggesting that PCR-errors had occurred during the initial amplification.

To assess the wider conservation of the *fHbp* PCR primers, six diverse *N. lactamica* isolates (Table 2-4) were tested in each PCR round individually. *N. lactamica* do not possess the *fHbp* gene, however, an allele encoding putative opacity protein (NLA18150) is found at this locus (Lucidarme et al., 2013a). The PCR primers target sites within flanking genes and should, therefore, yield a PCR product if the binding sites are conserved. For PCR round one, a PCR product was obtained from all six isolates, however, four of the six isolates failed to yield a product using the PCR round two primers. Analysis of the primer binding sites within the genomes of these four *N. lactamica* isolates revealed the identical *fHbpRd2R* mismatches as found in M11 240189 (Fig. 3-7).

For these four isolates, 2 µL of the amplified PCR round one product was used as a template for PCR round two reactions as performed in the nested protocol. This was to attempt to repeat the successful nested PCR amplification seen in clinical specimen M11 909694 despite the primer mismatches. All four isolates produced an appropriately-sized PCR product following round two of the nested protocol.

3.2 An analysis of fHbp peptide sub-variant distribution among non-culture confirmed IMD cases: 2011-2015

Declaration: Much of the work described in the following section was published in Clark et al., 2016 and parts of the following paragraphs have been amended from this publication. This article was written solely by me and all words used are my own.

3.2.1 Laboratory confirmed IMD cases: Jan 2011 to Dec 2015

From the 1st January 2011 to the 31st December 2015, PHE MRU confirmed 4090 IMD cases. Table 3-4 contains the number of culture and non-culture confirmed cases from each calendar year. Overall, a downward trend was observed in the total number of laboratory-confirmed cases over the five years studied.

Table 3-4: Number of laboratory-confirmed IMD cases in England and Wales by calendar year and confirmation method: 2011 to 2015.

Calendar Year (Jan-Dec)	No. of non culture-confirmed cases (% of total)	No. of culture-confirmed cases	No. of total cases (n)
2011	498 (50.9)	481	979
2012	382 (48.2)	410	792
2013	331 (42.7)	445	776
2014	264 (38.6)	420	684
2015	298 (34.7)	561	859
Combined	1773 (43.3)	2317	4090

The proportion of cases confirmed by PCR alone reduced yearly between 2011 and 2015. Over the five years, 43.3% of IMD cases were confirmed by PCR alone.

Table 3-5 illustrates the capsular group distribution among culture and non-culture cases.

Table 3-5: No and proportion of each capsular group within culture and non-culture cases for each calendar year.

Year	No. of (% of yearly) culture-confirmed cases						No. of (% of yearly) non culture-confirmed cases					
	B	Y	W	C	Other	NG	B	Y	W	C	Other	NG
2011	355 (73.8)	79 (16.4)	26 (5.4)	16 (3.3)	1 (0.2)	4 (0.8)	448 (90.0)	18 (3.6)	7 (1.4)	12 (2.4)	0	13 (2.6)
2012	278 (67.8)	69 (16.8)	38 (9.3)	18 (4.4)	1 (0.2)	6 (1.5)	347 (90.8)	14 (3.7)	6 (1.6)	10 (2.6)	0	5 (1.3)
2013	275 (61.8)	70 (15.7)	70 (15.7)	25 (5.6)	0	5 (1.1)	295 (89.1)	13 (3.9)	11 (3.3)	8 (2.4)	0	4 (1.2)
2014	220 (52.4)	66 (15.7)	105 (25.0)	27 (6.4)	0	2 (0.5)	215 (81.4)	11 (4.2)	19 (7.2)	11 (4.2)	1 (0.4)	7 (2.7)
2015	246 (43.9)	98 (17.5)	184 (32.8)	21 (3.7)	3 (0.5)	9 (1.6)	236 (79.2)	14 (4.7)	32 (10.7)	10 (3.4)	0	6 (2.0)
Combined	1374 (59.3)	382 (16.5)	423 (18.3)	107 (4.6)	5 (0.2)	26 (1.1)	1541 (86.9)	70 (3.9)	75 (4.2)	51 (2.9)	1 (0.4)	35 (2.0)

Group B accounted for most culture and non-culture cases with just under half of all group B cases yielding a culturable isolate (47.1%). The second largest group in both datasets was group W. A steep (650%) and steady increase in this group was seen between 2011 and 2015, however, only a small minority of these cases (15.1%) were confirmed by PCR alone. Group Y was the third most prevalent group, representing 11.1% of all cases. Group C represented only 3.9% of all cases. Other less common groups (e.g. A and E) and non-groupable strains were found among 1.6% of total cases.

Figure 3-8 shows the differences in method of confirmation among different age groups for each capsular group. The majority of all group B cases (57.2%) were seen among those aged four years or younger. For group W and Y disease, the largest numbers of cases were, however, seen in older individuals with 37.8% and 43.6% of cases among those >60 years of age, respectively. With regards to confirmation method, a similar overall pattern was seen across the three capsular groups with isolation of a viable organism more common among cases in young infants (<12 months) and older adults (>60 years).

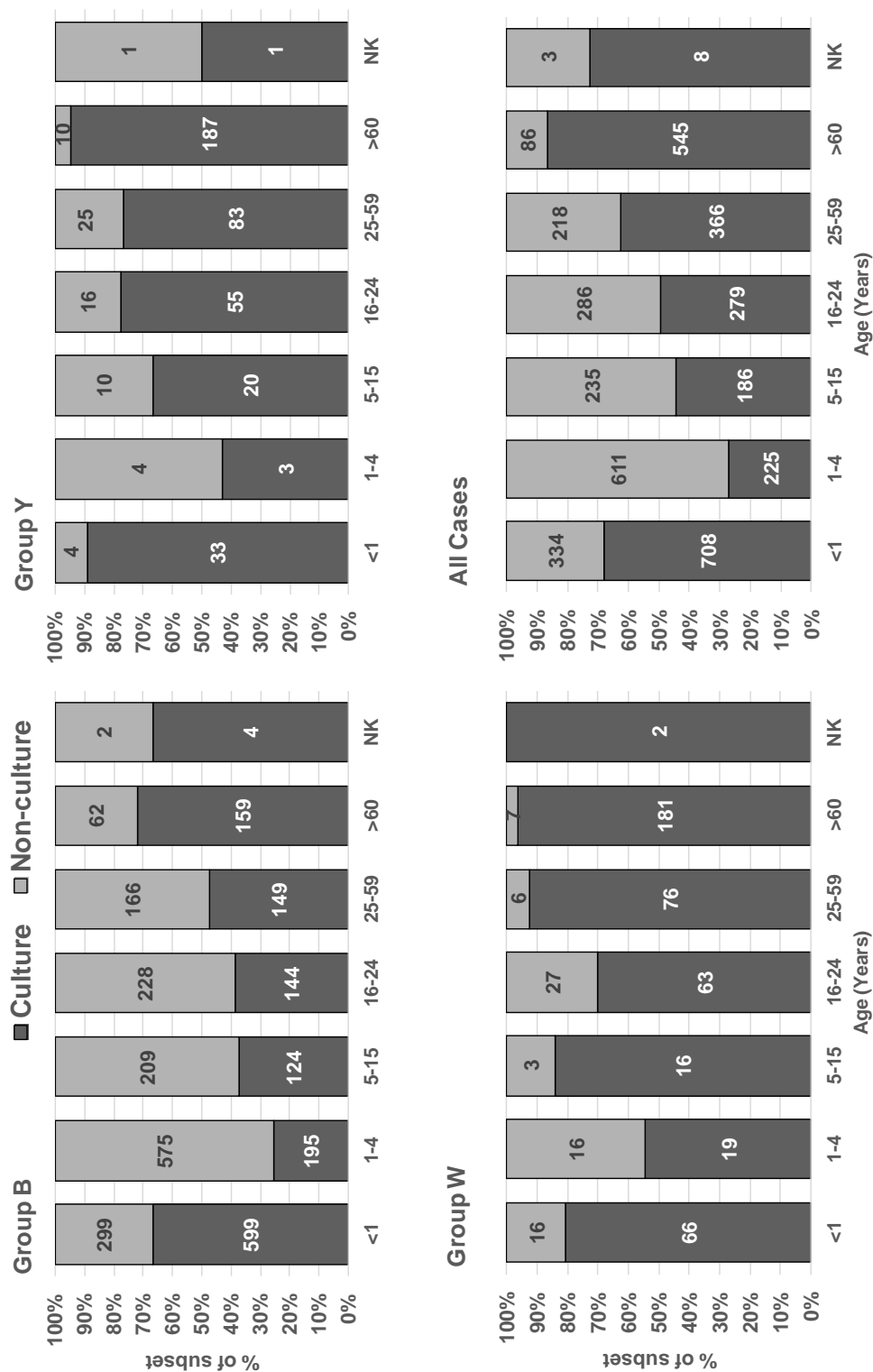


Figure 3-8: The proportions of culture and non-culture-confirmed group B, group Y, group W cases and all cases combined, stratified by patient age group. Numbers overlaid indicate numbers of cases within each subset. Dark grey bars represent the proportion of cases within each subset that were culture-confirmed. Light grey bars represent non-culture cases. NK=age of patient not known.

3.2.2 Overview of *fHbp* genotyping and variant group distribution.

Among all 2317 invasive isolates sequenced, 2304 (99.4%) possessed a full-length *fHbp* peptide. Ten isolates harboured alleles featuring frame-shift mutations which resulted in truncated alleles (nine ST-11 complex isolates harboured *fHbp* allele 669 and a ST-32 complex isolate possess allele 743). Three isolates during this period were *fHbp*-null (i.e. did not possess an *fHbp* allele). Table 3-6 shows the proportion of non-culture IMD cases in each calendar year for which sufficient DNA extract and/or clinical specimen was available for *fHbp* genotyping. Over the five year study period, testing was not possible on 112 (6.3%) of non-culture cases due to lack of material. The proportion of available material increased between 2011 and 2015.

Table 3-6: The number and proportion of non-culture IMD cases with sufficient DNA extract and/or clinical specimen available for *fHbp* genotyping by calendar year.

Year	Total no. of cases	No. of specimens/extracts available (% of total)	No. of successfully-typed cases (% of total)
2011	498	461 (92.8)	411 (82.5)
2012	382	345 (90.3)	319 (83.5)
2013	331	317 (95.8)	298 (90.0)
2014	264	252 (95.5)	235 (89.0)
2015	298	286 (96.0)	247 (82.9)
Combined	1773	1661 (93.7)	1510 (85.2)

Overall, a PCR product was successfully amplified and sequenced from 90.9% of the non-culture cases that had an available specimen and/or DNA extract. For each calendar year, these data represented between 80% and 90% of all non-culture IMD cases with 85.2% of non-culture cases typed over the five year period (Table 3-8).

Following the addition of the *fHbp* data from cultured isolates, of which all were successfully sequenced, a total of 93.6% (3827/4090) of all IMD cases confirmed from 2011 to 2015 were *fHbp* genotyped. Figure 3-9 shows the proportion of typed strains harbouring *fHbp* of each variant group among different capsular groups.

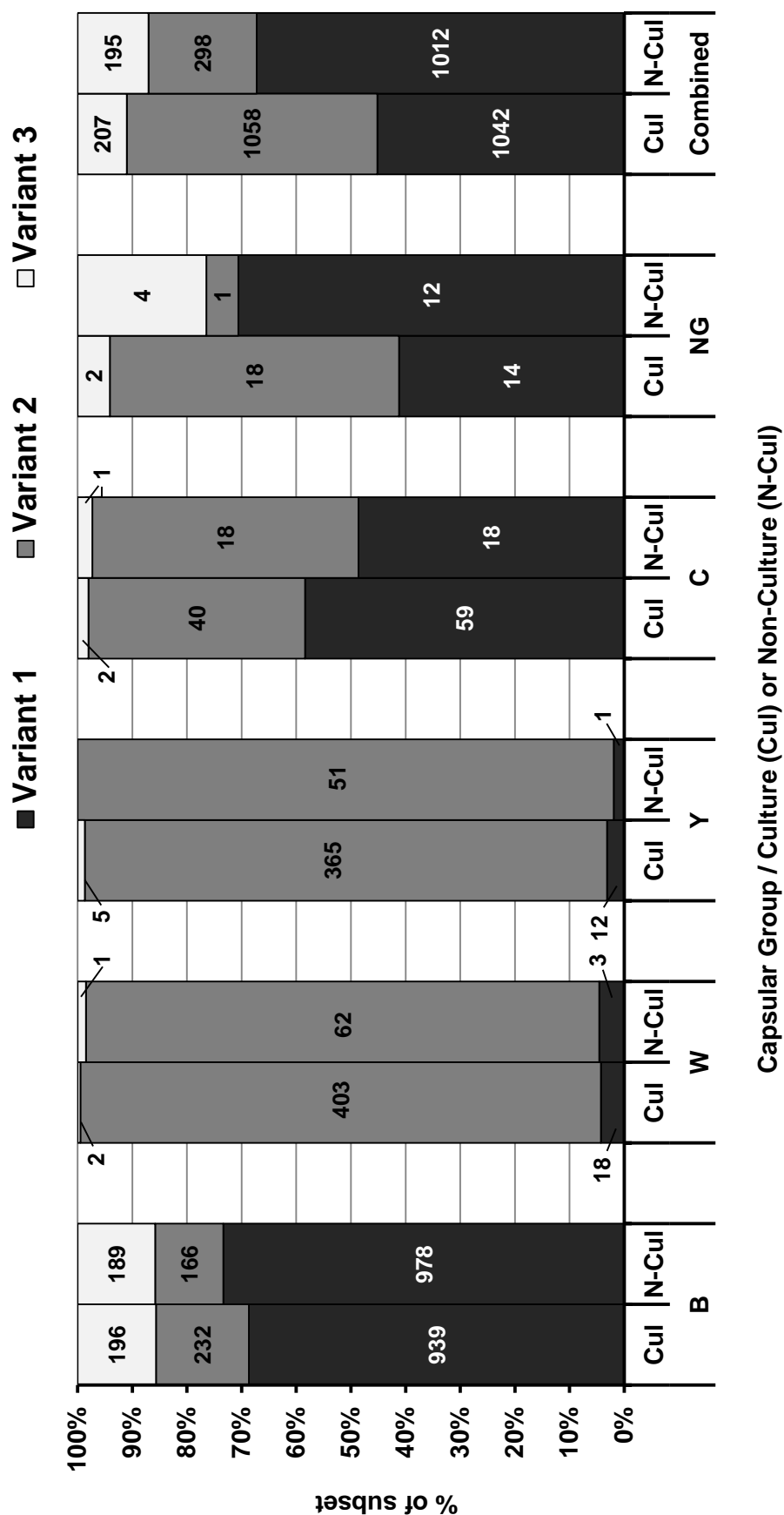


Figure 3-9: fHbp variant group distribution among typed culture and non-culture strains belonging to different capsular groups or non-groupable strains (NG). The corresponding numbers of strains are overlaid each bar. Strains that could not be characterised, harboured a truncated variant or were fHbp null (n=280) were omitted.

A noticeable disparity was observed in the prevalence of the different fHbp variant groups within strains of the different groups. Almost all characterised group W and Y strains harboured variant 2 fHbp peptides, whilst approximately two-thirds (70.9%) of group B strains possessed variant 1 peptides. Group C and non-groupable strains were also more commonly found to possess variant 1 peptides.

3.2.3 Distribution of common fHbp peptide variants

Among all characterised strains (n=3827), 305 unique *fHbp* alleles and 254 fHbp peptide variants were found. The following eleven variants, each represented by ≥ 50 strains, were found among 79.2% of all characterised strains: variants 1.4 (15.6%), 1.13 (12.3%), 2.22 (11.8%), 2.25 (10.1%), 1.15 (9.1%), 2.19 (5.9%), 1.14 (4.73%), 1.1 (3.5%), 3.45 (3.5%), 2.16 (2.6%) and 3.47 (1.7%). The majority of typed group W cases (82.8%) featured peptide variant 2.22. Variant 25 was predominant among group Y cases (83.4% of typed strains). Group C strains were more diverse with 1.13, 1.15, 1.19 and 2.22 collectively representing 71.2% of combined typed cases. Eight of the eleven common fHbp peptides mentioned above were present in substantial numbers among group B strains (each with ≥ 50 cases). Variants 1.1, 1.4, 1.13, 1.14, 1.15, 2.19, 3.45 and 3.47 collectively represented 75.1% of all typed group B strains.

Due to the homogeneity of group W and Y strains with respect to fHbp, there were no significant differences in the peptide distribution within these groups. Among group B cases, the proportional distribution of the eight common fHbp variants was relatively constant over the five years with a maximum variation of 1.04% (variant 1.13) to 5.2% (variant 15) of the total characterised group B cases. Fig. 3-10 shows the proportional changes in these eight variants among typed group B cases (n=2709) over the five years studied. Overall, the non-culture fHbp peptide distribution was similar to that seen among the isolates; however, consistent variation in the proportional distribution (across all five years) of three of the eight predominant variants (1.15, 2.19 and 3.47) was observed. Variants 2.19 and 3.47 were found more among the isolates than non-culture strains (Fig. 3-10), however, only the differences among strains harbouring 2.19 reached statistical significance (calendar year-adjusted Cochran-Mantel-Haenszel Test, $P=0.013$).

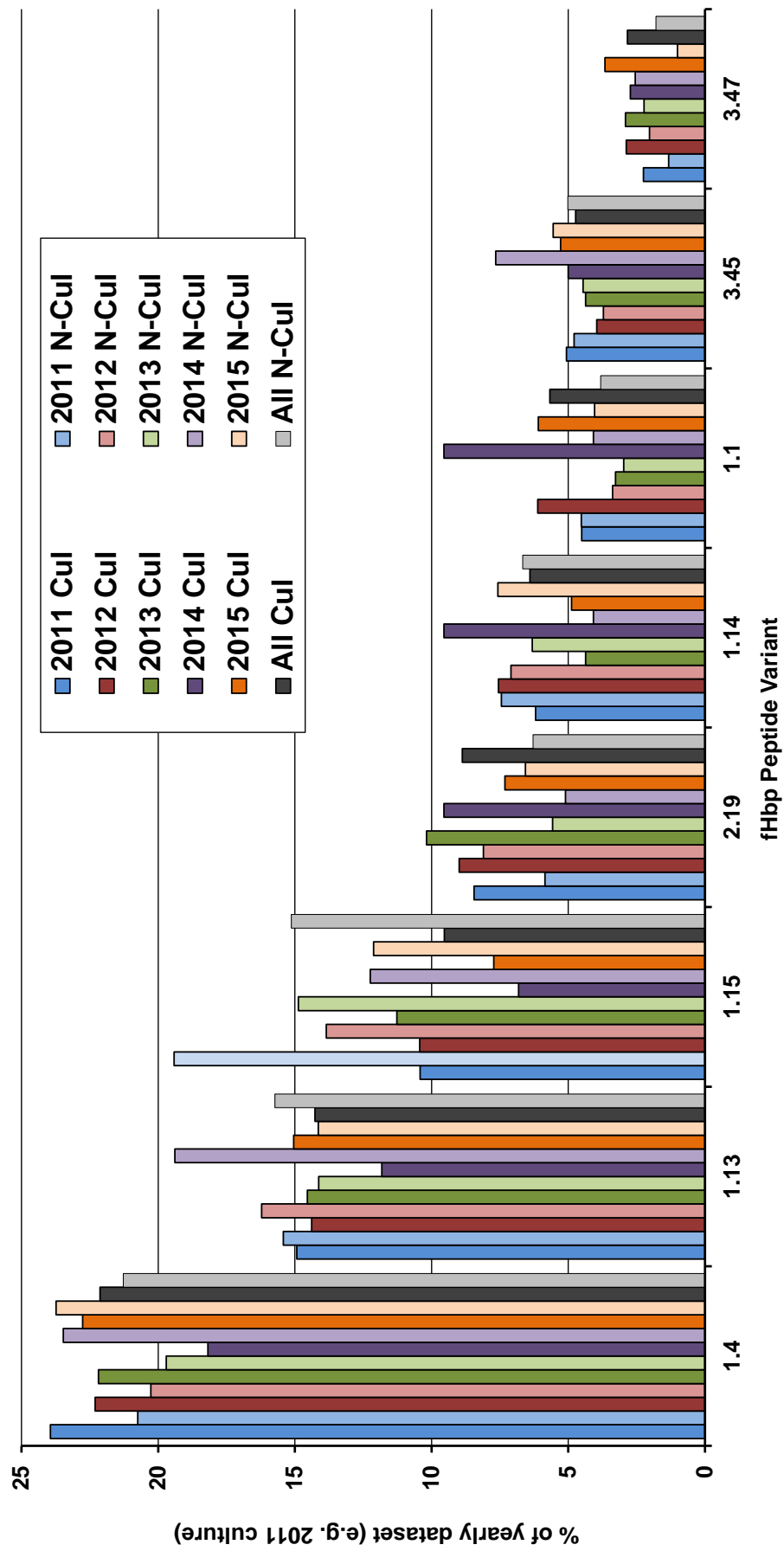


Figure 3-10: The proportion of culture or non-culture typed group B strains from each calendar year which are represented by eight common fHbp variants. Cul = Culture strains. N-Cul = Non-culture strains.

Peptide 1.15 was significantly more common among group B non-culture cases than isolates (calendar year-adjusted Cochran-Mantel-Haenszel Test, $P=0.000017$).

Stratification and analysis of the data by patient age groups revealed that these significant differences were restricted to those aged 16-24 years or 25-59 years (one-sided Fisher's exact tests, $P=0.007$ and $P=0.049$, respectively). Only characterised cases which had a known patient age were included ($n=2703$).

The peptide differences between culture and non-culture cases may be the result of features that are inherent to a subset of strains. Figure 3-11 shows the distribution of fHbp peptide variants among English and Welsh isolates received over six epidemiological years. The majority of the fHbp variants largely clustered within a single CC/cluster. Of the 252 isolates harbouring fHbp 1.15, 231 (91.7%) belonged to the ST-269 cluster. Isolates with peptide 2.19 were largely split between the ST-41/44 complex (27.6%) and the ST-275 cluster (51.6%).

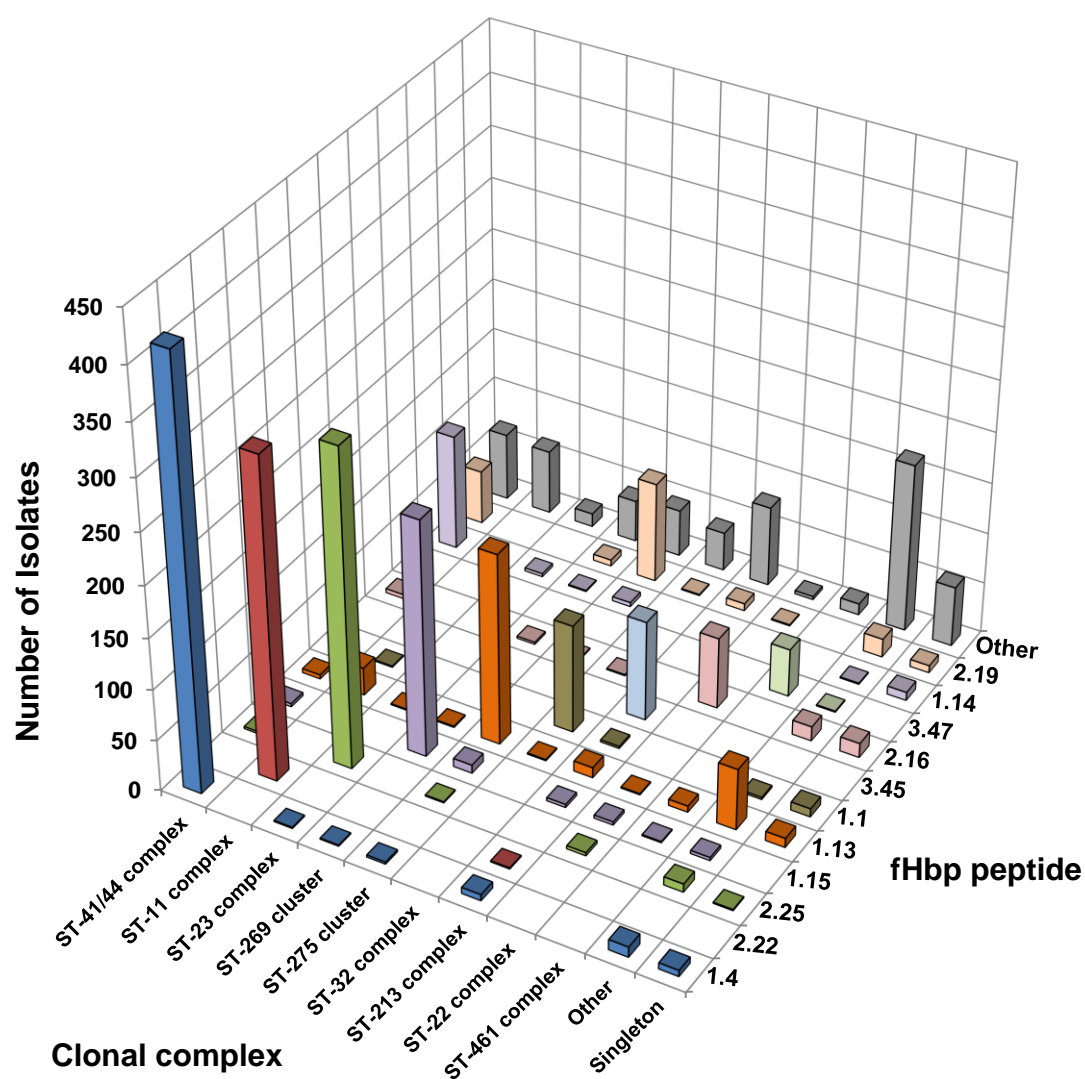


Figure 3-11: The distribution of fHbp variants among English and Welsh isolates by CC. Isolates received in 2007/08, 2010/11, 2011/12, 2012/13, 2013/14 and 2014/15 epidemiological years were included (n=2919). The ST-269 complex was divided into two subclusters: the ST-269 and ST-275 clusters. 'Singletons' refers to isolates with STs that are not part of a defined CC.

3.2.4 Representativeness of group B isolates

To assess how representative group B isolates are of group B disease as a whole (i.e. both cultured isolates and non-culture strains), a comparison of fHbp distribution among isolates and all cases combined was performed across different patient age groups.

Figure 3-12 illustrates the proportions of group B isolates and all cases represented by each of the common fHbp variants. The largest differences between isolates and all cases were observed for fHbp 1.15, with this variant representing 6.0% and 4.1% more of all cases than isolates alone within the 16-24 years and 5-15 years age groups, respectively. For all other variants and age groups, <3% differences were observed between isolates and all cases combined. When all ages were combined, the profile of group B isolates and all group B cases were very similar with no major differences other than that of variant 1.15.

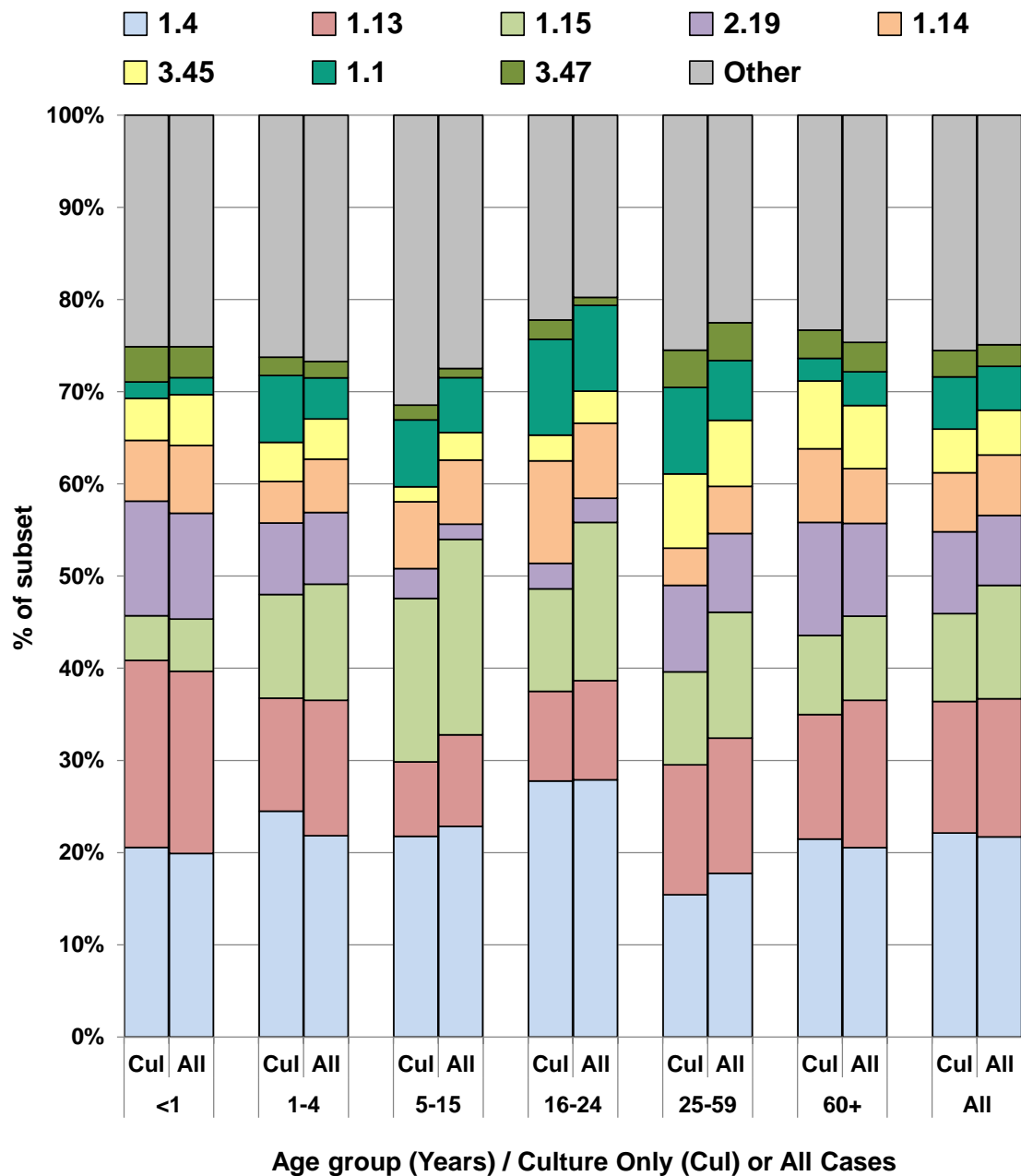


Figure 3-12: The representativeness of group B isolates amongst all typed group B strains in different age groups. Each bar represents the proportion of common fHbp variants within typed group B isolates (Cul, n=1374) and the corresponding proportions amongst all group B cases (culture and non-culture combined, n=2709) within the indicated age group or all ages.

3.3 Whole genome sequencing from non-culture specimens

Declaration: Much of the work described in the following section was published in Clark et al., 2017 and parts of the following paragraphs have been amended from this publication. This article was written solely by me and all words used are my own.

3.3.1 Specimen selection

The ten clinical specimens selected to evaluate the SureSelectXT Target Enrichment System as well as the MLST information of the corresponding isolates are listed in Table 3-7. The Ct values of the specimens ranged from 20-39. The meningococcal DNA concentrations of the specimens were calculated using the equation:

$y = 2 \times 10^7 e^{-0.589x}$ where Y equals the DNA concentration and x equals the Ct value. (Refer to section 2.8.2)

The estimated meningococcal DNA concentrations and corresponding total meningococcal DNA amounts are listed in Table 3-7 and ranged from 2 fg/μL to 153 pg/μL.

Table 3-7: Clinical specimens selected for evaluation of SureSelectXT system with corresponding clinical isolate information

Specimen ID	Specimen type	NM-specific real-time Ct value	Specimen volume (µL)	Estimated NM DNA concentration (pg/µL)	Estimated amount of NM DNA extracted (pg)	Corresponding isolate ID	Isolate ST	Isolate CC
M15 890512	Blood	39	200	0.002	0.423	M15 240120	2100	ST-213 complex
M15 890382	Blood	32	120	0.130	15.654	M15 240017	485	ST-41/44 complex
M15 901648	CSF	27	40	2.480	99.198	M15 240178	11481	ST-35 complex
M15 908607	CSF	27	55	2.480	136.398	M15 240270	11483	none
M15 894870	CSF	26	55	4.469	245.814	M15 240103	1946	ST-461 complex
M15 896440	Blood	28	300	1.376	412.826	M15 240142	749	ST-32 complex
M15 948231	CSF	26	120	4.469	536.322	M15 240805	213	ST-213 complex
M15 933165	Blood	24	100	14.516	1451.587	M15 240650	1049	ST-269 complex
M15 906731	CSF	23	75	26.160	1962.022	M15 240240	1161	ST-269 complex
M15 901404	Blood	20	300	153.123	45936.960	M15 240180	162	ST-162 complex

NM= *Neisseria meningitidis*

Samples of the two main types (blood and CSF) were selected from cases caused by diverse group B strains. A wide range of meningococcal DNA concentrations were selected using real-time PCR Ct values.

3.3.2 Genome coverage and depth of coverage of specimen-derived genomes

Table 3-8 lists the results of aligning the sequencing reads of specimen-derived genomes against the human reference genome (Hg19) and against the corresponding meningococcal pseudo-reference consensus.

Table 3-8: Results for sequencing of specimen-derived genomes and read alignment against human and meningococcal references.

Specimen ID	No. of reads pairs	No. of reads mapped to human ref. (Hg19)	% of reads mapped to human ref.	Closest-related complete genome	No. of reads mapped to NM pseudo-ref.	% of reads mapped to NM pseudo-ref.	% genome coverage \geq 1x	Median depth of coverage
M15 896512	1913131	1749684	91.5	alpha710	1112	0.1	7.2	0
M15 890382	1796259	1627329	90.6	NZ-05/33	22865	1.3	70.8	2
M15 901648	1769239	1518863	85.8	M04 240196	84281	4.8	93.7	10
M15 908607	1819385	1521127	83.6	153	124710	6.9	96.4	14
M15 894870	1932925	1635041	84.6	M22236	134565	7.0	92.4	17
M15 897440	1802516	1562163	86.7	MC58	91072	5.1	93.8	10
M15 948231	1959202	1070035	54.6	M01 240355	730805	37.3	98.1	89
M15 933165	2358677	964492	40.9	M04 240196	1250017	53.0	98.6	149
M15 906731	1594558	898513	56.3	M04 240196	563338	35.3	96.7	73
M15 901404	3276069	944227	28.8	12-176	2098332	64.1	95.6	254

NM= *Neisseria meningitidis*

For all ten samples, >90% of total reads aligned to either the human or meningococcal reference genome. The proportion of reads aligning to the human genome was generally inverse to the proportion aligning to the meningococcal reference. Eight of the ten specimen genomes exhibited >90% coverage and \geq 10 median read depth. These eight samples were estimated to contain >100pg of meningococcal DNA.

The proportion of reads aligning to the meningococcal reference ranged from 0.1% to 64.1%. The genome coverage of each samples ranged from 7.2% to 98.6%. The median depth of coverage varied from 0x to 254x.

All but two of the ten genomes yielded >92% genome coverage and ≥ 10 x median depth. Interestingly, for four of these eight high coverage specimens, <10% of the sequencing reads aligned to the meningococcal reference. Genome coverage values correlated positively with the estimated meningococcal DNA amount within the specimens, indeed all of the specimens with high genome coverage and depth contained an estimated ≥ 100 pg of meningococcal DNA (Table 3-8).

3.3.3 Gene-by-gene comparison of isolate and specimen-derived genomes

Table 3-9 contains meta-data on the paired (isolate and non-culture) genomes as well as the extracted typing data that are commonly used by reference laboratories. Of the ten specimen genomes, two did not yield recognisable meningococcal loci. Specimen M15 890512 was not accepted for submission to PubMLST (lowest DNA amount, 423 fg) and none of the indexed loci were identified within the genome of specimen M15 890382. The remaining eight specimens yielded genomes of acceptable quality with designated allele data for most of the loci of interest. When compared to the isolate genomes, the mean number of contigs of the specimen-derived genomes was substantially higher (1314 vs. 342) and among the eight acceptable genomes, the cumulative contig length was higher than for the corresponding isolate. This most likely indicates the presence of additional DNA sequences (host or contaminating bacteria) within the assembly.

Table 3-9: Genomic properties of specimen and isolate-derived meningococcal genomes and allelic data for common typing targets.

ID	DNA Source	No. of contigs	Cum. contig length	No. of tagged loci	PorA VR1	PorA VR2	PorB	FetA VR	fHbp peptide	NHBA peptide	NadA peptide	abcZ	adk	aroE	fumC	gdh	pdhC	pgm	ST	Clonal complex	Discrepant alleles/shared NEIS loci (n)
M15 890512	Specimen	889	574775																		
M15 240120	Isolate	342	2182243	1750	22	14	3-314	F3-6	45	18	0	7	5	1	13	9	53	9	2100	ST-213 complex	n/a
M15 890382	Specimen	1373	996602	0																	
M15 240017	Isolate	362	2101025	2000	12-1	16	3-817	F1-5	4	2	0	3	6	9	5	8	6	9	485	ST-41/44 complex	n/a
M15 901648	Specimen	1459	2363572	1193	21-2	28	3-45	F5-2	24		0	4	10	1	9	3	10	12	11481	ST-35 complex	8/1052
M15 240178	Isolate	344	2174757	1938	21-2	28	3-45	F5-2	24	29	0	4	10	1	9	3	10	12	11481	ST-35 complex	
M15 908607	Specimen	1224	2421445	1446	22	9	3-818	F1-8	13	24	0		5	34	17	3	172	124			
M15 240270	Isolate	302	2084949	1848	22	9	3-818	F1-8	13	24	0	12	5	34	17	3	172	124	11483	n/a	4/1287
M15 894870	Specimen	2449	2602857	1466	13-9	13-9	3-14	F3-9	47		0	12			35	192	22	17			
M15 240103	Isolate	399	2200706	1923	19-2	13-9	3-14	F3-9	47	118	0	12	5	12	35	192	22	17	1946	ST-461 complex	4/1318
M15 896440	Specimen	1509	2271736	1073	19	15	3-1	F5-1	1		1		10		4	6	3				
M15 240142	Isolate	315	2165187	1963	19	15	3-1	F5-1	1	120	1	8	10	77	4	6	3	8	749	ST-32 complex	11/945
M15 948231	Specimen	1307	2590449	1918	22	14	3-14	F5-5	185	18	0	7	5	1	13	36	53	15	213	ST-213 complex	8/1751
M15 240805	Isolate	315	2178381	1916	22	14	3-14	F5-5	185	18	0	7	5	1	13	36	53	15	213	ST-213 complex	
M15 933165	Specimen	1062	2469552	1981	19-1	15-11	3-423	F5-1	15	21	0	4	10	15	17	8	11	9	1049	ST-269 complex	2/1771
M15 240650	Isolate	420	2187886	1942	19-1	15-11	3-423	F5-1	15	21	0	4	10	15	17	8	11	9	1049	ST-269 complex	
M15 906731	Specimen	1079	2513378	1965	22	9	3-25	F4-1	13	17	0	4	10	34	5	38	11	9	1161	ST-289 complex	3/1780
M15 240240	Isolate	318	2162020	1956	22	9	3-25	F4-1	13	17	0	4	10	34	5	38	11	9	1161	ST-289 complex	
M15 901404	Specimen	789	2263139	1900	22	14	3-73	F5-9	700	20	0	1	5	13	53	26	41	3	162	ST-162 complex	3/1710
M15 240180	Isolate	305	2110985	1894	22	14	3-73	F5-9	700	20	0	1	5	13	53	26	41	3	162	ST-162 complex	

Filled dark gray boxes represent missing typing data

Specimens M15 890512 and M15 890382 did not yield identifiable meningococcal sequences. The common typing data extracted from the eight acceptable specimen genomes matched the corresponding isolate data. Genomes from specimens containing >500 pg of meningococcal DNA yielded valid allelic IDs for all common typing targets.

The eight acceptable specimen genomes produced allele IDs congruent with the corresponding isolate genomes at all of the characterised loci of interest. Whilst four of the eight acceptable specimen genomes yielded complete typing data, the remaining five genomes were missing data for at least one antigen and/or MLST loci (Table 3-9). A correlation could be observed between the estimated DNA amount within the specimen and the coverage of the loci of interest. Those specimen genomes that featured missing data all had predicted DNA amounts of <500 pg.

Following the expanded genome comparison at 2,652 indexed NEIS loci, substantial variation in the number of NEIS loci covered by both paired genomes in each case (Table 3-9). Between 945 and 1780 NEIS loci were present and complete in both genomes. The proportions of these that were discrepant were small, varying from 0.11% to 1.16%. The discrepant loci for each genome pair are listed in Appendix VI.

Of the genes with multiple nucleotide differences between paired genomes, all but one exhibited evidence of paralogy and/or produced significant additional hits during BLAST searches of complete meningococcal genomes. For the remaining gene, NEIS0534 (*rpsP*), four nucleotide differences were observed between the two genomes (specimen M15 908607 and isolate M15 240270). The four synonymous differences were within a 28bp region in the first half of the gene. BLAST searches of human genomes with NCBI Genbank yielded no significant hits.

3.3.4 Predicted utility of SureSelect XT system for characterisation of non-culture cases confirmed in England and Wales.

These data indicate that specimens containing ≥ 100 pg of meningococcal DNA are likely to yield genomes of high coverage and those with ≥ 500 pg of available target DNA appear to provide complete common typing data. To predict the proportion of non-culture cases confirmed by PHE MRU that contain sufficient target DNA, the hypothetical DNA amount within specimens over a wide range of *ctrA*-specific Ct values (19 to 40) and possible sample volumes was estimated (Table 3-10). The predicted likelihood of attaining an acceptable genome using the SureSelect XT system was determined in each case.

These predictions indicate that specimens producing Ct values >30 are unlikely to produce a genome of acceptable quality at any specimen volume (maximum extraction volume: 300 μ L). Specimens generating Ct values ≤ 26 were estimated to yield acceptable genomes from volumes down to only 25 μ L. Those with Ct values of ≤ 23 contain at least 500 pg and would be likely to yield better quality genomes (more complete loci). Between Ct values of 26 and 31, the likelihood of producing an acceptable genome is determined by the volume of sample available (Table 3-10).

From January 2016 to December 2016, PHE MRU confirmed 336 non-culture cases. As some cases yielded multiple specimens, the total number of PCR positive non-culture specimens was 495. Of all the PCR positive non-culture specimens, 54.5% (270/495) produced a Ct value of >30 and are, therefore, unlikely to generate an acceptable genome. After removal of duplicate specimens and selection of those with the lowest Ct value (i.e. one specimen per case), 156/336 (46.4%) produced Ct values of >30 . Approximately 28.3% (n=95) and 13.1% (n=44) produced Ct values of ≤ 26 and ≤ 23 , respectively. There were 85 non-culture cases for which the most concentrated specimen produced Ct values from 27 to 30 (25.3%) and so the likelihood of successful WGS would be dependent on remaining specimen volume.

Table 3-10: Estimated meningococcal DNA load of non-culture specimens and predicted outcome of non-culture WGS.

Ct value	Estimated NM DNA concentration (pg/μL)	Specimen volume (μL)											
		25	50	75	100	125	150	175	200	225	250	275	300
40	0.0012	0.0293	0.0586	0.0879	0.1172	0.1465	0.1759	0.2052	0.2345	0.2638	0.2931	0.3224	0.3517
39	0.0021	0.0528	0.1056	0.1585	0.2113	0.2641	0.3169	0.3697	0.4226	0.4754	0.5282	0.5810	0.6338
38	0.0038	0.0952	0.1904	0.2856	0.3808	0.4759	0.5711	0.6663	0.7615	0.8567	0.9519	1.0471	1.1423
37	0.0069	0.1715	0.3431	0.5146	0.6862	0.8577	1.0293	1.2008	1.3724	1.5439	1.7155	1.8870	2.0586
36	0.0124	0.3092	0.6183	0.9275	1.2367	1.5458	1.8550	2.1642	2.4733	2.7825	3.0916	3.4008	3.7100
35	0.0223	0.5572	1.1143	1.6715	2.2287	2.7859	3.3430	3.9002	4.4574	5.0145	5.5717	6.1289	6.6861
34	0.040	1.004	2.008	3.012	4.017	5.021	6.025	7.029	8.033	9.037	10.041	11.045	12.050
33	0.072	1.810	3.619	5.429	7.238	9.048	10.858	12.667	14.477	16.287	18.096	19.906	21.715
32	0.130	3.261	6.523	9.784	13.045	16.306	19.568	22.829	26.090	29.351	32.613	35.874	39.135
31	0.235	5.877	11.755	17.632	23.510	29.387	35.265	41.142	47.019	52.897	58.774	64.652	70.529
30	0.424	10.592	21.184	31.777	42.369	52.961	63.553	74.145	84.738	95.330	105.922	116.514	127.106
29	0.76	19.09	38.18	57.27	76.36	95.45	114.53	133.62	152.71	171.80	190.89	209.98	229.07
28	1.38	34.40	68.80	103.21	137.61	172.01	206.41	240.81	275.22	309.62	344.02	378.42	412.83
27	2.48	62.00	124.00	186.00	248.00	310.00	371.99	433.99	495.99	557.99	619.99	681.99	743.99
26	4.47	111.73	223.47	335.20	446.93	558.67	670.40	782.14	893.87	1005.60	1117.34	1229.07	1340.80
25	8.05	201.36	402.73	604.09	805.46	1006.82	1208.19	1409.55	1610.92	1812.28	2013.65	2215.01	2416.38
24	14.5	362.9	725.8	1088.7	1451.6	1814.5	2177.4	2540.3	2903.2	3266.1	3629.0	3991.9	4354.8
23	26.2	654.0	1308.0	1962.0	2616.0	3270.0	3924.0	4578.1	5232.1	5886.1	6540.1	7194.1	7848.1
22	47.1	1178.6	2357.3	3535.9	4714.6	5893.2	7071.9	8250.5	9429.1	10607.8	11786.4	12965.1	14143.7
21	85.0	2124.1	4248.3	6372.4	8496.5	10620.7	12744.8	14868.9	16993.1	19117.2	21241.3	23365.5	25489.6
20	153.1	3828.1	7656.2	11484.2	15312.3	19140.4	22968.5	26796.6	30624.6	34452.7	38280.8	42108.9	45937.0
19	276.0	6898.9	13797.8	20696.7	27595.6	34494.5	41393.5	48292.4	55191.3	62090.2	68989.1	75888.0	82786.9
18	497.3	12433.1	24866.2	37299.3	49732.5	62165.6	74598.7	87031.8	99464.9	111898.0	124331.1	136764.3	149197.4
17	896.3	22406.8	44813.6	67220.3	89627.1	112033.9	134440.7	156847.4	179254.2	201661.0	224067.8	246474.5	268881.3
16	1615.2	40381.2	80762.3	121143.5	161524.6	201905.8	242287.0	282668.1	323049.3	363430.5	403811.6	444192.8	484573.9
15	2911.0	72774.3	145548.7	218323.0	291097.3	363871.7	436646.0	509420.4	582194.7	654969.0	727743.4	800517.7	873292.0
14	5246.1	131152.8	262305.7	393458.5	524611.4	655764.2	786917.1	918069.9	1049222.7	1180375.6	1311528.4	1442681.3	1573834.1
13	9454.5	236361.7	472723.5	709085.2	945446.9	1181808.6	1418170.4	1654632.1	1890993.8	2127255.6	2363617.3	2599797.0	2836340.7
12	17038.7	425967.6	851935.3	1277902.9	1703870.6	2129838.2	2555805.8	2981773.5	3407741.1	3833708.7	4259676.4	4685644.0	5111611.7
11	30706.9	767672.6	1535345.3	2303017.9	3070690.5	3838363.1	4606035.8	5373708.4	6141581.0	6909053.7	7676726.3	8444398.9	9212071.5
10	55339.5	1383488.3	2766976.7	4150465.0	5533953.4	6917441.7	8300930.1	9694418.4	11067906.8	12451395.1	13834883.5	15218371.8	16601860.2

Red filled: <100 pg, predicted to be insufficient for genome sequencing.
Yellow filled: 100-500 pg, predicted to be sufficient for genome sequencing but may feature missing typing data.
Green filled: >500pg, predicted to be sufficient for genome with complete typing data.
NM= *Neisseria meningitidis*

Predicted DNA loads were calculated based on an estimated DNA concentration (determined by Ct value) and a hypothetical specimen volume. Specimens with Ct values >26 are predicted to contain >100pg of DNA, even if only 25 μL of specimen is available for extraction. At Ct value 30 and above, 300 μL of sample is predicted to contain <100 pg. For the currently used extraction protocol (Qiagen, UK), 300 μL is the maximum volume used.

3.4 Transfer of the MEASURE Assay

3.4.1 Assessing killing effectiveness of 1% PFA/PBS

During the MEASURE assay, PFA/PBS is used to fix meningococcal cells prior to staining (McNeil et al., 2018). This process is important to ensure the cells are non-viable and that the biological risk associated with meningococcal cultures is eliminated. The original MEASURE protocol specified that the cells were incubated in PFA/PBS for at least ten minutes. As part of the transfer of the assay to PHE MRU, an assessment of the ability of PFA/PBS to kill the meningococcal cultures was performed.

The initial killing assessment involved testing four strains with three different PFA/PBS batches using the “dilution” method (refer to 2.12.1) at four time points: 15 minutes, 30 minutes, 45 minutes and 60 minutes. The initial test (using batch PFA007) showed survival of three of four strains after 45 minutes incubation and one strain survived up to 60 minutes (Table 3-11). Control plates (incubated in PBS without PFA) showed confluent growth.

To confirm the results, the test was repeated using a second PFA/PBS batch (PFA008) for up to 80 minutes. All four strains survived up to forty minutes incubation. Two strains survived for 60 minutes and M01 240070 survived for 80 minutes (Table 3-12). Control plates showed confluent growth. To ensure these results were not due to a defective 16% PFA batch, a new PFA/PBS batch (PFA009) was prepared from a different 16% PFA batch. Two of the four previously used strains were tested against PFA009 and were both shown to tolerate 30 minutes (Table 3-13). M01 240070 was again the most tolerant and survived 60 minutes in the new batch. Control plates showed confluent growth.

Table 3-11: Meningococcal growth following up to 60 minutes incubation in 1% PFA.

Isolate ID	1% PFA Batch	No. of colonies after x minutes in 1% PFA			
NZ98/254	PFA007	14	0	1	0
M01 240070		Conf.	>200	>200	>100
M04 241215		4	1	1	0
M01 240601		0	0	0	0

Table 3-12: Meningococcal growth following up to 80 minutes incubation in second 1% PFA batch.

Isolate ID	1% PFA Batch	No. of colonies after x minutes in 1% PFA			
NZ98/254	PFA008	30	3	0	0
M01 240070		60	40	30	3
M04 241215		12	2	0	0
M01 240601		14	4	2	0

Table 3-13: Meningococcal growth following up to 60 minutes incubation in third 1% PFA batch.

Isolate ID	1% PFA Batch	No. of colonies after x minutes in 1% PFA			
M01 240070	PFA009	~200	~100	44	12
M04 241215		~50	10	0	0

These initial results illustrated that ten minutes incubation is not sufficient to kill all meningococcal strains and at least one strain can survive up to 80 minutes.

After the initial results, changes were made to the assessment methodology in order to increase sensitivity. The next assessment involved significantly extended time points including overnight incubation (≥ 16 hours). Using the newly-developed centrifugation method (refer to 2.12.2), the two strains tested exhibited survival after 4 hours incubation in PFA/PBS (Table 3-14). Control plates showed confluent growth.

Table 3-14: Meningococcal growth following up to 20 hours incubation in PFA011. No growth was observed at 16 or 20 hours time points.

Isolate ID	1% PFA Batch	Number of colonies after x hours in 1% PFA			
		3 hours	4 hours	16 hours	20 hours
M01 240007	PFA011	~300	~200	0	0
M01 240070		~300	~200	0	0

Following overnight incubation at 16 and 20 hours, no growth was observed for either strain. The overnight assessment (16 hours incubation) was repeated with six additional, diverse strains using two additional PFA/PBS batches (Table 3-15). Whilst one PFA/PBS batch killed all six strains (PFA012), the other (PFA013) failed to kill all but one strain after 16 hours. Control plates showed confluent growth.

Table 3-15: Meningococcal growth following 16 hours incubation in PFA012 and PFA013. No growth was observed after 16 hours in PFA012 for any strain; however six strains survived 16 hours in PFA013.

Isolate ID	1% PFA Batch	Number of colonies after 16 hours in 1% PFA
M15 240133	PFA012	0
	PFA013	35
M04 240731	PFA012	0
	PFA013	~200
M05 240300	PFA012	0
	PFA013	0
M14 240606	PFA012	0
	PFA013	5
M15 240465	PFA012	0
	PFA013	5
M14 240616	PFA012	0
	PFA013	30
M15 240098	PFA012	0
	PFA013	~200

To confirm these results and determine whether PFA013 may be a defective batch, the two most tolerant strains from the preceding assessment were tested once again against PFA013 as well as five new batches, PFA014 to PFA018. After 16 hours, PFA013 once again failed to kill both strains, however, no growth was observed after incubation in any of the five new PFA/PBS batches (Table 3-16). Control plates showed confluent growth.

Table 3-16: Meningococcal growth following 16 hours in five newly-prepared PFA/PBS batches. All PFA batches but PFA013 killed both strains after 16 hours incubation.

Isolate ID	1% PFA Batch	Number of colonies after 16 hours in 1% PFA
M04 240731	PFA013	3
	PFA014	0
	PFA015	0
	PFA016	0
	PFA017	0
	PFA018	0
M15 240098	PFA013	23
	PFA014	0
	PFA015	0
	PFA016	0
	PFA017	0
	PFA018	0

Finally, in order to determine the shelf life of the PFA/PBS, selected batches were tested after different intervals. Table 3-17 shows the results of three batches tested following 11 weeks storage at 4°C and one batch 21 weeks after preparation. All batches were effective against both strains tested after 16 hours incubation. Control plates showed confluent growth. These results suggest that PFA/PBS batches are likely to retain killing effectiveness after at least 21 weeks of storage at 4°C.

The findings of this killing assessment resulted in changes being made to the MEASURE protocol in order to accommodate an overnight incubation step (≥ 16 hours) prior to antibody staining of fixed meningococcal cells. Whilst these changes altered the days on which certain stages of the assay were performed, no further alterations to the protocol were made. The new assay protocol was successfully re-validated by the assay developers and no significant impact on the results was observed following introduction of the overnight incubation (data not available).

Table 3-17: Meningococcal growth following 16 hours in 11 week-old or 21 week-old PFA/PBS. No growth was recorded for either strain after 16 hours in any of the PFA batches.

Isolate ID	1% PFA Batch	Number of colonies after 16 hours in 1% PFA
M04 240731	PFA016*	0
	PFA017*	0
	PFA018*	0
	PFA019 [#]	0
M15 240098	PFA016*	0
	PFA017*	0
	PFA018*	0
	PFA019 [#]	0

* 11-weeks since preparation, [#] 21-weeks since preparation

3.4.2 Assessing the impact of agar type used during production of GC glycerol broth

During the set-up of the assay, it quickly became apparent that the GC agar used for initial cultivation of the organisms was not sensitive enough to support sufficient growth unless a highly-concentrated inoculum was used. As such, inoculating the GC agar plates directly from microbank beads was not possible. Meningococcal strains had to be prepared in GC glycerol broths prior to testing. In the assay, the organism is grown overnight on agar prior to preparation of the GC glycerol broths. Due to the greater financial cost and diameter of the GC agar, using CBA for this step was deemed preferable. To assess any potential impact of using CBA as opposed to GC agar, a comparison of MEASURE MFI results between strains grown from GC glycerol broths produced using the different agars was performed.

Nine control strains were tested using both GC glycerol broth types using the finalised MEASURE protocol. For the GC glycerol broths produced using GC agar, ten MFI values were generated over the course of the assay transfer. Only two MFI results were generated for each of the GC glycerol broths grown from CBA. The results are shown in the Figure 3-13.

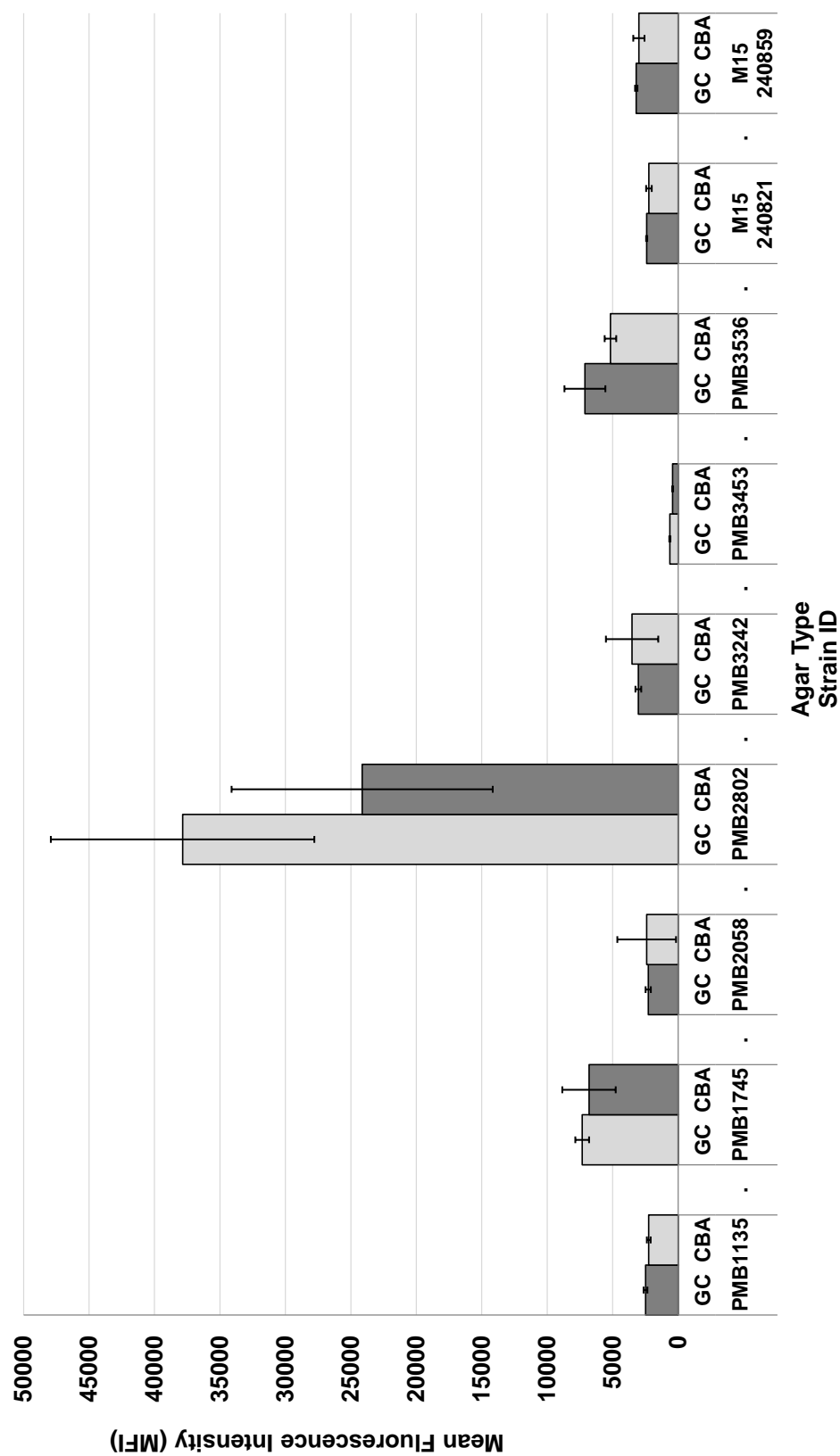


Figure 3-13: Mean MEASURE MFI values of nine strains when the GC glycerol broth was produced from overnight grown on GC and CBA agar. Error bars indicate 95% confidence intervals. The results were similar between all strains grown on either the GC and CBA agar. A number of strains showed wide confidence intervals as a result of small sample sizes.

The mean MFI results were very similar between the GC glycerol broths produced using either agar type with overlapping 95% confidence intervals for all strains. The sample size was, however, low and so it is difficult to accurately assess the impact of this change.

3.4.3 Inter-laboratory comparison of MEASURE controls

A comparison of MEASURE MFI results generated between PHE MRU and a collaborating institution was performed. Seven control strains were tested six times in duplicate, thus generating twelve MFI values (Table 3-18).

Table 3-18: MFI values, means and 95% confidence intervals (CI) for strains tested using MEASURE assay at PHE MRU. The MFI values varied between strains but were consistent across all replicates.

	Strain ID						
	PMB 1135	PMB 1745	PMB 2058	PMB 2802	PMB 3242	PMB 3453	PMB 3536
1	2602	7242	1841	57694	3111	712	8081
2	2400	6515	1874	56885	3127	654	7329
3	2878	8075	2304	38596	3010	648	6742
4	2488	8940	2423	32653	2861	639	7172
5	2785	7472	2391	50464	2784	560	10543
6	2414	7295	2194	46025	2653	612	10823
7	2353	7133	2523	23291	2896	624	5179
8	2326	6389	2282	26628	3700	703	4962
9	2230	6967	2578	24921	3672	420	5208
10	2394	7286	2799	23811	2893	524	5423
11	2233	6914	2201	22461	3365	652	4926
12	2254	6646	2225	23351	3358	414	5138
Mean	2446.4	7239.5	2302.9	35565.0	3119.2	596.8	6793.8
95% CI	2312.9 - 2579.9	6792.6 - 7686.4	2129.6 -2476.2	26794.1 - 44335.9	2903.3 - 3335.1	534.0 - 659.7	5453.3 - 8134.4

Mean MFI values varied widely between strains from 597 (PMB3453) to 35,565 (PMB2802). Within strains, the values were quite consistent, however, only PMB2058 was normally distributed around the mean.

Figure 3-14 illustrates the comparison of the data generated at PHE MRU with the equivalent data generated in the collaborating laboratory. The data from the collaborators were generated over 24 runs (96 data points), by three operators and using two cytometers (both BD Accuri C6). Whilst the median values were reasonably similar for each strain, those generated at PHE MRU were higher than those from the collaborating laboratory across all seven strains. Furthermore, all but one of the strains showed statistically significant differences between the laboratories (Mann-Whitney test, $p < 0.011$). The strain producing congruent inter-laboratory values, PMB3453, was the lowest expressing strain. When commercial phycoerythrin (PE) reagent beads (Bangs Laboratory Inc., US) were used to assess and compare cytometer performance, the cytometer at PHE MRU generated mean MFI values that were 12% higher than those at the collaborating laboratory (data not available).

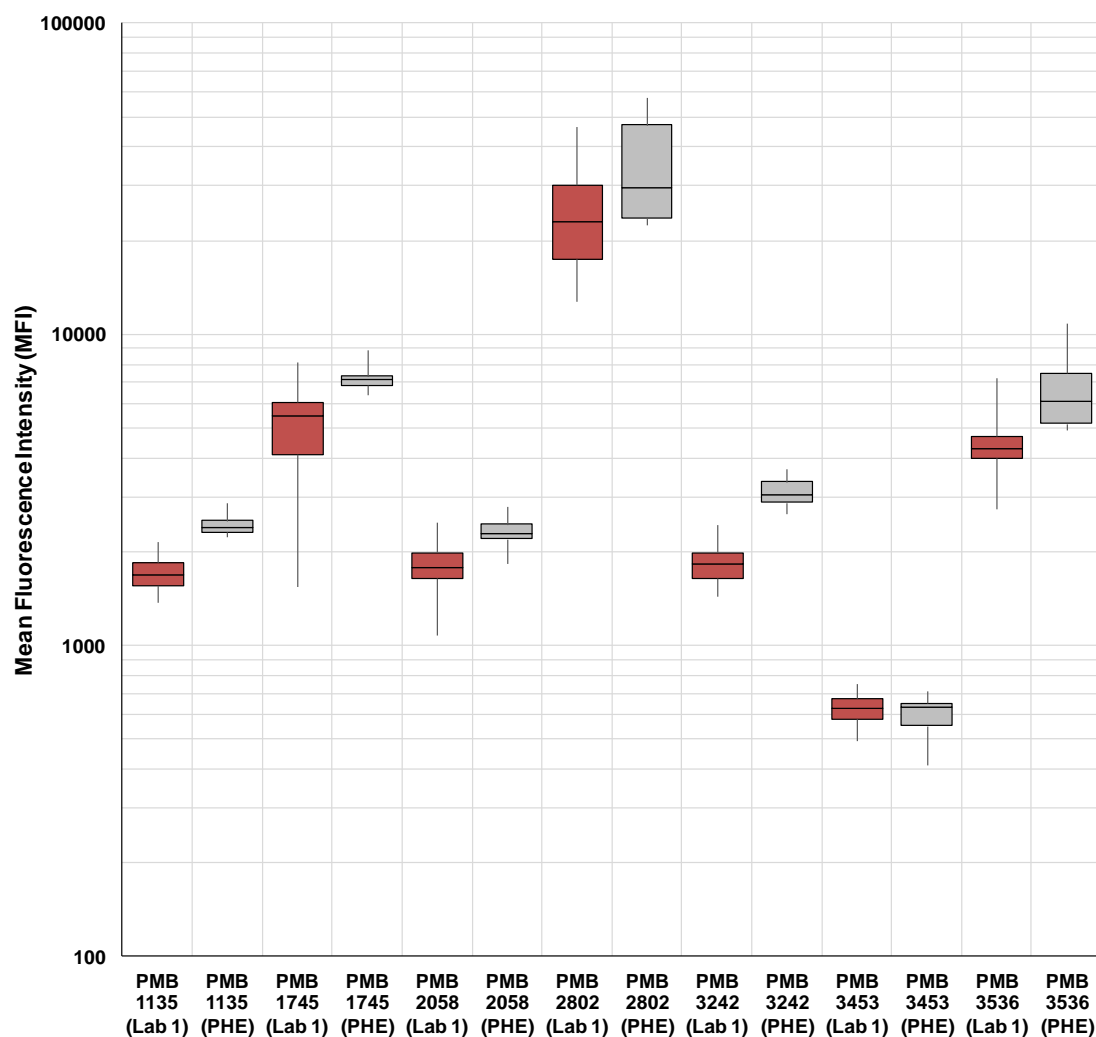


Figure 3-14: Box and whisker plots showing comparison of MEASURE results between PHE MRU and a collaborating laboratory (Lab 1) for seven control strains. The upper and lower box limits indicate the 75% and 25% quartiles, respectively, whilst the central line denotes the median values in each case. The whiskers illustrate the maximum and minimum values.

4.0 DISCUSSION

Since 2012, two protein-based group B meningococcal vaccines have been licenced for use in Europe and beyond. One of these vaccines, 4CMenB, was introduced into the UK national infant immunisation schedule in late 2015 alongside the commencement of an Enhanced Surveillance programme in order to assess the impact of this intervention. Key to this programme, PHE MRU performs strain characterisation on submitted clinical isolates and residual meningococcal DNA from within clinical specimens. Assessing the strain coverage of these vaccines is a large part for this characterisation and the work described herein involved the development and/or assessment of new assays which will aid ongoing strain coverage assessments of 4CMenB and future assessments of other protein-based vaccines.

4.1 Development and validation of *fHbp* PCR sequencing assay

To facilitate the development of an *fHbp* nested PCR assay, existing *fHbp* primers were assessed to determine their suitability for use in the assay. One set of existing primers (1869-2F and 1871Ralt) were determined to be suitably conserved and had previously been shown to be effective in studies of fHbp distribution in meningococci and the presence of *fHbp* in *N. lactamica* (Lucidarme et al., 2013a, 2011a).

Two new PCR primers, *fHbpRd2F* and *fHbpRd2R*, were designed for the second PCR round of the nested protocol. Optimisation experiments using genomic DNA as a template (i.e. not nested PCR product) showed positive amplification at a wide range of annealing temperatures (51-63 °C). A very small amount of non-specific product was visible on the gel at all temperatures tested, however the use of a nested protocol may help to reduce these products by increasing the specificity. Similarly, using different primers for sequencing should help prevent any impact on the sequencing data. Whilst the highest annealing temperature (63 °C) was selected for the standard PCR protocol, the ability to use a lower annealing temperature maybe useful in future in order to compensate for any primer-binding site mismatches.

After testing a panel of 96 diverse clinical isolate/specimen pairs, all isolates were successfully amplified using the round one primers. An *fHbpRd2R* binding site

mismatch in one of the 96 isolates prevented amplification using the round two primers (not nested). This mismatch was found to be very uncommon among invasive meningococci within the MRF MGL, but was observed among four of the sixteen *N. lactamica* genomes studied. The mismatch could conceivably become more common due to natural fluctuation in strain distribution or horizontal gene transfer amongst *Neisseria* strains. Interestingly, however, the use of a nested PCR protocol allowed amplification of the second round product. The precise mechanism through which the primer binding site mismatch was countered was not determined, but it is hypothesised that the greater amount of DNA template during round two of a nested protocol (i.e. round one product compared to genomic DNA template during a single PCR) may increase the chances of semi-complementary binding of the mismatched primers during the early PCR cycles. The resultant products would feature the exact primer sequence, allowing efficient amplification in the subsequent cycles. For this reason, *fHbpRd2R* was deemed acceptable for use in the assay. The use of a nested protocol may also compensate for any other primer mismatches in future.

Upon sequencing of the amplified extracts, two of the isolate/sample pairs had *fHbp* alleles that were discrepant at one base pair. Re-amplification and sequencing of *fHbp* from the clinical specimen extracts yielded the matching alleles indicating that the initial mismatch was due to a PCR error. These findings suggest that samples that yield novel *fHbp* alleles, especially those which are only one base pair different from an indexed allele, should be re-amplified and sequenced in order to prevent false alleles being included in the PubMLST database.

In order to assess the sensitivity of the newly-developed assay, a panel of serially diluted DNA extracts from diverse isolates was tested using the finalised nested protocol. Using the standard 10 µL of DNA template in a 50 µL round one reaction, the nested assay successfully amplified extracts down to an estimated meningococcal DNA concentration of 600 ag/µL. The sensitivity could be increased to a DNA concentration of ~6 ag/µL in some cases by using 20 µL of extract in a 100 µL reaction.

This sensitivity level was then correlated to the Ct values generated using the *ctrA*-specific Taqman® assay in order to predict the proportion of clinical specimens which

could be sufficiently typed. As the assay format had recently moved from duplex, wet-mix assay to a quadruplex, lyophilised mix assay, the diluted extracts were tested using both. The lyophilised Taqman® assay exhibited lower sensitivity in relation to the wet mix assay, especially at lower DNA concentrations. This could be due to the differences in the sensitivities of the fluorophores used for the *ctrA* probes (6-FAM and LIZ for wet mix and lyophilised assay, respectively). For the lyophilised assay, the additional group B *siaD* target does, however, provide supplementary detection in the majority of cases (i.e. group B cases). The DNA extracts of one of the isolates produced consistently higher Ct values than the other isolates in both the wet and lyophilised assays. This is most likely due to an error during quantification and/or dilution.

The results of the optimisation and Taqman® experiments suggest that using 5 µL or 10 µL of DNA extract in round one of the nested assay should allow amplification of *fHbp* from samples that produced Ct values up to 41 using lyophilised Taqman® assay (~600 ag/µL). In 2011/12, 98% of the non-culture samples received produced a Ct value of ≤41 (unpublished data, PHE MRU). Using 20 µL of extract would increase the sensitivity further, and would theoretically allow amplification of some extracts that may have produced a negative *ctrA* result in the lyophilised assay (or Ct 42 using wet mix assay).

Although using greater volume of extract slightly increases the sensitivity of the assay, the clinical specimens from which the DNA is extracted are in most cases unreplenishable. The extracts should, therefore, be conserved as much as possible for typing of other targets and/or future work. Using greater extract volume also involves using a greater total reaction volume which leads to increased reagent costs. For these reasons, 10 µL of extract in a 50 µL reaction volume was chosen for the finalised PCR round one protocol. Another way of conserving extract/reagents would be to restrict the use of a nested PCR to the weaker samples only. The validation data indicate that the template concentration that generates visible amplification after only a single PCR round (using 5 µL of extract) corresponds to a *ctrA* Ct value of ~30-32. It could, therefore, be suggested that those clinical extracts that produce Ct values of ≤32 should be tested using 5 µL of extract in a non-nested *fHbp* PCR protocol.

It must be noted that these data are based on analyses of diluted purified meningococcal extracts and not clinical extracts which contain human DNA as well as potential PCR inhibitors which may impact the sensitivity of the Taqman® and/or *fHbp* typing assay. The true sensitivity of the assay could only be determined by testing a large number of clinical extracts.

4.2 An analysis of fHbp peptide sub-variant distribution among non-culture confirmed IMD cases: 2011-2015

Following the development of a sensitive *fHbp* PCR sequencing assay, it was applied to clinical specimens from non-culture IMD cases confirmed over five calendar years (2011-2015). The total number of non-culture cases reduced between 2011 and 2015, which is consistent with the gradual reduction in group B cases observed in previous decade (Table 1-4). The increase in group W disease somewhat compensated for this reduction since 2012 and contributed to the small increase in total cases in 2015. The proportion of cases confirmed solely by molecular methods also reduced steadily between 2011 and 2015. This can largely be attributed to the increase of group W disease over this period, of which only a small proportion were confirmed by PCR. The disparity among group B cases and group W and Y cases with regards to method confirmation is attributed to the differences in patient age distribution between the strains (Ladhani et al., 2012a). It is hypothesised that among older patients with possible co-morbidities and/or non-specific symptoms (e.g. pneumonia and septic arthritis), meningococcal disease is less likely to be suspected by clinicians and, as a result, the rate of submission of clinical specimens for meningococcal PCR testing is much lower than for younger patients presenting with archetypal meningitic/septicaemic symptoms. This of course suggests there could be a substantial number of IMD cases within these older age groups that are escaping laboratory confirmation leading to an under-estimation of the total disease burden.

In 2011, due to a relatively small proportion of DNA extracts available for testing, DNA from 89.1% of the specimens was manually re-extracted using the Qiagen Blood and Tissue kit. For subsequent years, however, changes in practices pertaining to the retention of the extracts within PHE MRU led to an increase in the number of extracts

available, negating the use of secondary DNA extraction. Over the five years, 93.7% of all non-culture cases had an extract available for testing and 85.2% were successfully characterised. This represents a substantial proportion of cases and provides a reasonable overview of fHbp distribution.

The subfamily distribution of the fHbp variants varied significantly between strains of different capsular groups with almost all groups W and Y featuring subfamily A variants and two-thirds of group B strains harbouring variants of subfamily B. This is consistent with studies in other countries (Wang et al., 2011; Law et al., 2014). There was only slight variation in subfamily distribution between culture and non-culture strains within each group. There was substantial difference in subfamily distribution when all groups were combined, however, this is largely due to the differences in capsular groups between culture and non-culture (i.e. fewer group W and Y among non-culture strains). In terms of specific fHbp peptide variants, group W and Y strains were very homologous with the vast majority of all strains harbouring 2.22 or 2.25, respectively. This reflects the homogeneity of these strains in terms of CC distribution. The majority of group W isolates belonged to either ST-22 complex or ST-11 complex, whilst almost all of the group Y strains belong to ST-23 complex. Group B strains possessed a greater array of fHbp variants reflecting the greater diversity of these strains.

After a comparison of group B isolates and non-culture strains, two of the eight commonly-observed variants were found to be consistently greater in one of the data sets than the other in all five calendar years. Variant 2.19 was significantly more common among isolates. The reason for the difference is not known, however, increased viability of the organism during infection and *in vitro* would explain this observation. As fHbp plays a key role in protecting the strain against the host complement system, it is conceivable that expression of a specific variant with high fH affinity could mediate such an increased viability. Although, a recent study of *fHbp* promoter regions found that strains harbouring variant 2 fHbp peptides are likely to share a common fHbp promoter clade which is associated with low surface expression (promoter clade V) (Biagini et al., 2016).

In contrast, fHbp 1.15 was found to be significantly more common among non-culture cases. This may indicate a lack of viability or an increased susceptibility to antimicrobial treatment. Unlike 2.19, which is associated with multiple common CCs, 1.15 was found almost exclusively among ST-269 cluster strains. This could suggest the presence of a characteristic unique to this population that is responsible for this pattern. In a recent study, variant 1.15 had a ten-fold lower affinity for fH than 1.1 and expression of 1.15 was associated with lower growth in blood and plasma. It must be noted, however, that a correlation between fH affinity and meningococcal survival in blood, plasma or serum was not clearly demonstrated (Dunphy et al., 2011; Seib et al., 2011). Another recent study revealed that ST-269 cluster strains lack NalP, a serine protease autotransporter which is prevalent in most other predominant group B lineages (Oldfield et al., 2013). Among a large collection of both carriage and invasive isolates, the *nalP* gene had been deleted in all ST-269 cluster isolates, whilst such deletion was only observed sporadically in other group B strains. NalP has been shown to increase the survival of meningococci in serum by cleaving complement component 3 (C3), NHBA and Lactoferrin Binding Protein B from the membrane surface (Serruto et al., 2010; Roussel-Jazédé et al., 2010; Del Tordello et al., 2014). It is conceivable therefore that the lack of NalP may therefore result in a lower growth and/or survival rates of invading ST-269 cluster strains *in vivo*, reducing the likelihood of bacterial isolation.

The difference between fHbp 1.15 prevalence among culture and non-culture was largely restricted to older children, adolescents and young adults. This is consistent with the hypothesis that ST-269 cluster strains are more susceptible to clearance, as individuals in these age groups are likely to have better-developed immune responses than infants and young children. The age-specific pattern may also be due to strain-independent factors such as differences in disease presentation which could possibly affect diagnostic practices (as suggested for group W and Y strains).

The final analysis assessed the representativeness of group B isolates among all group B strains. The proportions of cases represented by the common fHbp variants were very similar between isolates and all cases, especially when looking at all ages combined. Within specific age groups, the aforementioned differences in subvariant 1.15 prevalence were notable. These results suggest that, overall, cultured group B isolates

can be relied upon to provide a representative sample of all English and Welsh invasive group B strains. Culture-based assessment of vaccine antigens such as those undertaken in the MATS and MEASURE assays are, therefore, likely to provide accurate estimations of vaccine strain coverage. It must be acknowledged, however, that this study only focussed on one sub-capsular antigen and that currently unseen differences between culture and non-culture strains may be revealed following more expansive characterisation, such as whole genome sequencing.

4.3 DNA enrichment and genomic analysis of non-culture IMD specimens

Despite increasing practicability in recent years, whole genome analysis of meningococcal strains has largely been restricted to cultured isolates (Tagini and Greub, 2017). The ability to sequence meningococcal genomes directly from clinical specimens has been hindered by low target DNA content and the presence of non-target DNA, primarily human host DNA. As up to a half of IMD cases in England and Wales are confirmed without strain isolation, this restriction severely limits the extent of meningococcal strain characterisation in England and Wales as a whole.

Whole genome analysis of non-culture cases would support the enhanced surveillance of vaccine antigens and investigations into potential vaccine failures. In recently studies, genomic data have been used to predict vaccine antigenic expression levels, identify newly-emerging invasive sub-lineages and provide epidemiological links between invasive strains during outbreaks (Chatt et al., 2014; Tzeng et al., 2017; Biagini et al., 2016; Lucidarme et al., 2015). The DNA within these specimens represents a genetic ‘snap shot’ mid-infection. As such, performing WGS on these materials may improve our understanding of meningococcal virulence.

Target specific oligonucleotides have been used to enrich DNA from a wide array of sample types in a number of diverse fields including environmental microbiology and archaeology (Carpenter et al., 2013; Vezzulli et al., 2017). The Agilent SureSelectXT system has been previously used to sequence the genomes of herpesvirus, norovirus *Chlamydia trachomatis* and *Mycobacterium tuberculosis* directly from clinical specimens (Nimmo et al., 2017; Depledge et al., 2011; Christiansen et al., 2014; Brown

et al., 2015, 2016). In this study, eight of the ten IMD specimens selected yielded draft genomes of acceptable coverage and depth. For the remaining two specimens, the lack of genomic data generated is highly likely to be due to a dearth of meningococcal DNA. Other studies using the SureSelectXT system on bacterial targets have been successful, however, the success rate is difficult to compare as different targets have widely varying genome sizes (e.g. *C. trachomatis* has genome size of ~1 Mbp whilst *M. tuberculosis* has a genome size of ~4.4 Mbp) and the samples from which the DNA was extracted varied (e.g. vaginal swabs, sputum, blood etc.) (Christiansen et al., 2014; Brown et al., 2015; Tagini and Greub, 2017).

After comparing the specimen-derived genomes to the genomes of corresponding isolates, the isolate-derived genomes contained fewer contigs and, in most cases, a greater number of annotated genes. This finding generally indicates that the isolate genomes are of relatively better quality, although this is somewhat unsurprising given the source. The eight, acceptable specimen-derived genomes featured a greater cumulative contig length in comparison to the corresponding isolates. This suggests that additional, non-target sequences (human and/or contaminating DNA) were included in the assembly despite the enrichment and screening of the reads for human sequences.

Comparisons of the typing data extracted from the paired specimen/isolate genomes revealed perfect agreement in terms of the allelic IDs of commonly-used typing loci, although some genes were incomplete in genomes derived from specimens with <500 pg of meningococcal DNA. Among a wider panel of NEIS loci (n=2652), only a small number of discrepant genes were identified, which illustrates the accuracy and reliability of the enrichment process. Most of the discrepancies could be explained as mis-assembly of the reads due to paralogous sequences. For one discrepant gene, *rpsP* (NEIS0534), no evidence of paralogy/presence of similar sequences could be identified. All four nucleotide differences were synonymous (therefore unlikely be influential) and within a short region of the gene. Thus far, an explanation for these differences is not forthcoming; however, characterisation of more specimen-derived genomes in future may yield an explanation.

Whilst the RNA bait sequences were generated from a large number of genomes (n=2975), *N. meningitidis* exhibits high homologous recombination rates, which may hinder the cross-reactivity of the baits and less common invasive strains or carriage strains (Vos and Didelot, 2009). In this study, these RNA baits appeared to be effective at enriching DNA from diverse group B strains, however, the wide array of DNA amounts among the selected specimens made it difficult to compare the hybridisation efficiency among different strains. In addition, the isolate and specimen genomes were produced at different times, using different assembly/sequencing methods. This is a limitation of the comparison and makes it difficult to accurately assess the influence of DNA target enrichment on sequence quality.

The results generated from this small study suggest that specimens containing ≥ 100 pg of meningococcal DNA are likely to yield genomes of sufficient quality. Based on these data, it was estimated that 28.3 to 53.6% of the non-culture IMD cases confirmed by PHE MRU could be whole genome sequenced using this technique. Whilst this would represent a significant increase in the total proportion of E&W invasive meningococcal strains from which genomic data are available, it is likely that approximately one quarter of all strains from laboratory confirmed IMD cases would remain largely uncharacterised. It must, however, be noted that this study represents a first attempt. Future optimisation of DNA extraction protocols and/or pre-enrichment depletion of non-target DNA have been shown to improve genome quality (Brown et al., 2015). Such optimisations may also increase the proportion of non-culture IMD cases which can be whole genome sequenced.

One of the most important factors influencing the utility of new techniques/assays is the financial cost and, in this case, the use of the SureSelectXT system is likely to be 2-3x the cost per strain than the corresponding protocols for whole genome sequencing from cultured isolate DNA. Whilst this does represent a significant outlay and may preclude the application of this technique to all eligible non-culture cases, when comparing this cost to the current non-culture characterisation activities (i.e. *fHbp* and PorA VR non-culture genotyping), the SureSelectXT system is likely to be substantially more efficient in terms of the number of loci characterised per pound sterling and per microlitre of sample/extract. Although reagent costs are substantially higher than for

traditional PCR and Sanger sequencing, consolidation of molecular characterisation to a single protocol would prove to be a significant saving in terms of laboratory time and staff costs. Nonetheless, the cost may still be too high at present to justify WGS of all non-culture cases. As such, analyses of specific samples of interest (e.g. outbreak samples) may be more practical.

4.4 Assessment and transfer of the MEASURE assay

The MEASURE assay is a flow cytometry-based assay used to quantify the amount of surface-expressed fHbp on fixed meningococcal cells. The assay was developed and validated in a collaborating laboratory and was transferred to the PHE MRU for independent assessment of UK and European meningococcal strains. Previous studies have shown a strong positive correlation between MEASURE MFI and the SBA activity of rLP2086 antisera (Jiang et al., 2010; McNeil et al., 2018).

The assay involves the cultivation of relatively large volumes of liquid meningococcal culture, which represents a significant risk to the operator and other laboratory staff. As part of the transfer, a comprehensive risk assessment was performed. One of the key elements of the risk assessment was to confirm that the fixing method utilising 1% PFA/PBS was sufficient to render the cultivated cells non-viable and eliminate the biological risk further downstream in the assay. PFA is a commonly used fixative and has advantages over other fixatives (e.g. solubility, cost, protein cross-linking). The results of a number of experiments indicated that the original assay protocol, involving incubation of cells in PFA/PBS for at least 10 minutes, was grossly insufficient to render the cells non-viable.

The collaborating laboratory indicated that the killing assessments initially carried out relied on GC agar to test for meningococcal survival (personal correspondence). It was later confirmed that GC agar is significantly less sensitive at growing meningococci than blood-based agar. This is an effect that has been observed in previous studies which utilised GC-based agar (personal correspondence, Dr. Steve Gray PHE MRU).

Using a sensitive recovery method, it was determined that some meningococcal strains can survive up to and possibly beyond four hours in PFA/PBS. The time points were

therefore extended (16 and 20 hours) to assess survival after an overnight PFA/PBS incubation. All nine of the diverse strains tested were non-viable after 16 hours using seven different PFA/PBS batches. Unfortunately, one batch, PFA013, was ineffective after 16 hours suggesting that PFA013 was a defective batch. The reason for this is unclear. Whilst unlikely, perhaps not all of the 16% PFA was added to the PBS diluent in error. Alternatively, the formaldehyde within the 16% ampoule could have polymerised and thus reducing the concentration of the solution. Whatever the cause, this result supports the adoption of a validation step to ensure the effectiveness of new 1% PFA/PBS batches prior to use.

As a result of these experiments, the assay was altered to allow for an overnight (≥ 16 hours) incubation. This necessitated assay re-validation by the collaborating laboratory. The re-validation confirmed that extension of the incubation duration does not impact on the results of the assay.

Further validation activities included comparing the use of GC agar and CBA for production of GC glycerol broths. The use of GC glycerol broths was required as GC agar does not sufficiently support growth when inoculated directly from microbank beads. The limited tests performed indicated broad similarity between the final MEASURE MFI results when the GC glycerol broth was prepared by either agar. Whilst the sample size was small and further tests are required to confirm these findings, they suggest that culturing methods prior to assay day one do not influence fHbp expression. Previous work which was carried out in attempt to bridge the assay to using solid agar showed that changes to the agar during the MEASURE assay itself (assay day one) can influence fHbp expression values (Clark et al., 2013).

Once the assay had been fully established within the laboratory, several preliminary runs were performed using seven control strains. The results for each strain appeared to be reasonably consistent over six assay runs; however, a comprehensive assessment of assay repeatability was not performed.

Finally, a comparison of the assay results generated at PHE MRU and those produced by collaborating laboratory revealed similar but significantly different results for all but the low-expressing control strain (PMB3453). The PHE MRU generally produced higher

MFI values than the counterpart laboratory. These differences could be caused by a myriad of factors including variation among operators, reagents and/or equipment. Many of the assay-specific reagents (e.g. media and antibodies) were provided directly by the sponsor and attempts were made to ensure additional, non-supplied reagents (e.g. PBS, paraformaldehyde, BSA) are standardised as much as possible. It is, therefore, more likely that the differences were due to operator and/or equipment variation.

In the MATS assay, reference strains are used to generate standard curves against which test strains are compared (Donnelly et al., 2010). Presumably, this internal comparator acts to reduce inter-operator and inter-laboratory variation. Indeed, such analyses have revealed high concordance between MATS results generated in multiple laboratories (Plikaytis et al., 2012). Despite the use of positive control strains, no such internal reference is used to calculate the readout in the MEASURE assay. The assay result is therefore more susceptible to variation that will inevitably occur as the assay is performed by different operators, using different equipment in different laboratories. In 2017, rLP2086 was licenced in Europe for use in those aged ≥ 10 years. Although it is not currently used routinely in the UK, it could be utilised in future to control outbreaks in adult populations (e.g. universities and colleges). In this circumstance, the MEASURE assay would provide a key indicator of its probable strain coverage. Consequently, any variation in the MEASURE readout as a result of operator/equipment changes could influence the likelihood of the vaccine being used.

The MATS assay is also used to assess the level of fHbp expression. Although they both quantify fHbp expression, the MEASURE assay differs from the MATS assay in several important ways. The use of a cross-reactive monoclonal antibody in the MEASURE means that the expression values should be independent of the peptide variant being quantified, unlike MATS which uses polyclonal antibodies specific to the 4CMenB vaccine variant (1.1). Whilst the ability of MN86-994-11-1 to bind diverse fHbp variants has been illustrated in previous studies (and indeed in the current results), data on potential variation in affinity between different fHbp variants is limited. McNeil et al. (2018) found that the binding affinities of MN86-994-11-1 were quite consistent across eight diverse fHbp peptides representing different subfamilies ($K_D = 10^{-10}$ to 10^{-12}),

however, it is conceivable that fHbp variants with significantly different MN86-994-11-1 affinities could exist among the invasive population (McNeil et al., 2018). This study identified the key binding residues and assessed the sequence variation among this small number of fHbp peptides, however, a more comprehensive comparison of these residues among a much wider array of fHbp peptides (e.g. all peptides in PubMLST database) would be a relatively simple undertaking and would provide greater confidence in the consistency of MN86-994-11-1 and the MEASURE assay as a whole.

Despite these uncertainties, using a monoclonal antibody in the MEASURE assay provides a more objective assessment of fHbp expression than the MATS, which is will only be relevant in the context of 4CMenB. The MEASURE is likely to be a very useful tool for assessing expression of variant groups 2 and 3 (which cannot be measured using MATS), elucidating the mechanisms of fHbp expression and monitoring changes in fHbp expression among predominant strains over time.

The MEASURE differs from MATS in that it quantifies only surface-expressed fHbp, whilst the MATS quantifies total cellular fHbp protein. It could be argued that, because immune recognition relies upon surface expression, the MEASURE provides a more relevant measure of expression. The relationship between *fHbp* gene transcription, peptide translation and translocation to the bacterial surface has not been extensively studied. In 2016, Biagini *et al.* measured total fHbp protein levels among diverse strains using quantitative mass spectrometry. Using these values and a standard bacterial surface area value (derived from MC58), they calculated an estimated number and density of fHbp molecules on the surface, assuming fHbp is equally distributed. These calculations assumed that all fHbp was located on the cell surface, however, this was not confirmed. McNeil et al. (2018) used MEASURE and Western blotting to suggest that a MEASURE MFI of 1000 is equivalent to 30 pg of fHbp protein per μg of cellular protein, although this again does not provide a precise quantification of fHbp expressed on the surface (McNeil et al., 2018). It may be possible that, much like fHbp expression generally, the level of translocation of translated fHbp peptide to the surface varies between strains. If this is the case, flow cytometry-based assays like the MEASURE would provide a more relevant prediction of serum bactericidal responses.

4.5 Conclusions and further work

The primary purpose of this work was to improve and expand the methods employed by the PHE MRU in order to characterise invasive meningococcal strains. In September 2015, the 4CMenB sub-capsular meningococcal vaccine was introduced into the UK infant immunisation schedule. In order to assess the impact of the vaccine, an Enhanced Surveillance programme was launched which utilises many different characterisation methods (Parikh et al., 2017). Whole genome sequencing and MATS testing provide broad characterisation of meningococcal isolates and can accurately predict whether cultured strains are expected to be covered by the vaccine post-implementation (Medini et al., 2015). For 40-50% of IMD cases, however, no isolate is obtained. Previously, characterisation of these strains was limited to genogrouping and geno-subtyping (PorA sequencing). As 4CMenB is not a polysaccharide-based vaccine, genogrouping provides no definitive indication of coverage. PorA sequencing does allow assessment of coverage of the OMV component, however, only 16% of E&W isolates in 2014/15 harboured the vaccine variant, P1.4 (Parikh et al., 2017). The development of the *fHbp* genotyping assay represented an expansion of the partial ability to assess 4CMenB coverage among non-culture strains, whilst providing an almost complete strain coverage prediction for rLP2086, which contains only fHbp. The ability to assess expression, however, is currently not possible without a viable isolate. Although, the PCR product amplified during the non-culture assay contains the complete intergenic region upstream of *fHbp*. As such, only minor changes to the sequencing protocol would be required in order to sequence this promoter as well as the coding region. The ability to accurately predict the level of fHbp surface expression directly from promoter sequences has not yet been convincingly demonstrated, however, strong correlations between specific promoter clades and high or low expression levels have been reported (Biagini et al., 2016). In the near future, it may be possible to make reliable predictions of fHbp expression without the need to test a viable isolate *in vitro*. This would allow comprehensive non-culture strain coverage predictions for fHbp, which will help to better assess 4CMenB going forward and provide more accurate predictions of rLP2086 coverage.

Much of the work has focussed on fHbp, however, the two other recombinant protein antigens (NadA and NHBA) are likely to offer protection, in particular NHBA which was predicted to be protective against 34% of E&W strains collected in 2014/15 (Parikh et al., 2017).

Characterisation of these antigens among isolates is achieved using WGS and the development of WGS protocols for non-culture clinical samples represents a big step forward in terms of improving strain coverage assessments. The SureSelectXT system is currently predicted to be effective for up to 54% of non-culture cases, however, further optimisation is required. The column-based DNA extraction technique currently used has a maximum sample volume which restricts the amount of DNA which can be extracted. Investigations into alternative methods (e.g. bead-based methods) could improve the proportion of strains which can be tested.

Other DNA enrichment techniques could also be investigated, including the generation of oligonucleotide baits in-house and hybridisation of native DNA fragments instead of DNA libraries (Tsangaras et al., 2014; Gasc and Peyret, 2017). The generation of baits in-house would likely reduce costs but could also allow the production of more specific baits by utilising the vast library of meningococcal DNA stored within PHE MRU (e.g. generation of baits specific to certain capsular groups). Enrichment of native, non-fragmented DNA would allow for sequencing using platforms that produce longer read lengths, e.g. PacBio RSII (Nakano et al., 2017). Such technologies improve assembly of difficult regions (e.g. repeat motifs, paralogous loci), and could conceivably improve genome coverage in relation to sequencing of DNA libraries. Other techniques such as selective whole genome amplification (SWGA) could also be assessed (Clarke et al., 2017). In SWGA, a set of small oligonucleotides are designed *in silico* and used in a multiple displacement amplification reaction in order to preferentially amplify target genomes in the presence of background genomes. It has been shown to be effective at amplifying DNA from mixed clinical samples in order to sequence *M. tuberculosis* and *Plasmodium vivax* with high genome coverage (Cowell et al., 2017; Clarke et al., 2017).

For now, however, comprehensive strain coverage assessments, especially those incorporating measurements of fHbp expression are limited to cultured meningococci. The analysis of fHbp variants among five years worth of non-culture samples did, however, indicate that isolates do provide a reasonable representative sample of all invasive strains, at least within capsular groups. Slight variation was observed in particular fHbp variants among culture and non-culture strains but whether these differences persist into the future remains to be seen. Non-culture *fHbp* genotyping continues to be performed routinely as part of the Enhanced Surveillance programme and future comparisons are likely to be made.

In conclusion, the assays described in this thesis will help to improve the accuracy of post-licensure assessment of 4CMenB. Strain coverage represents a key aspect in determining vaccine effectiveness and impact and these activities will help to influence decisions on future utilisation of 4CMenB, as well as rLP2086 and future sub-capsular meningococcal vaccines.

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6.0 APPENDICES

Appendix I: The isolates used to determine the conservation of the *fHbp* primer sites and to identify prospective primer candidates.

No.	Isolate ID	No.	Isolate ID	No.	Isolate ID	No.	Isolate ID	No.	Isolate ID	No.	Isolate ID	No.	Isolate ID	No.	Isolate ID	No.	Isolate ID
1	M10 240473	44	M10 240638	87	M11 240025	130	M11 240071	173	M11 240216	216	M11 240352	259	M11 240491	302	M11 241043	345	M12 240261
2	M10 240474	45	M10 240640	88	M11 240026	131	M11 240072	174	M11 240227	217	M11 240358	260	M11 240492	303	M11 241044	346	M12 240262
3	M10 240478	46	M10 240641	89	M11 240027	132	M11 240073	175	M11 240231	218	M11 240358	261	M11 240493	304	M11 241046	347	M12 240263
4	M10 240480	47	M10 240642	90	M11 240028	133	M11 240074	176	M11 240233	219	M11 240360	262	M11 240592	305	M11 241047	348	M12 240267
5	M10 240481	48	M10 240643	91	M11 240029	134	M11 240100	177	M11 240236	220	M11 240363	263	M11 240594	306	M11 241048	349	M12 240272
6	M10 240482	49	M10 240649	92	M11 240030	135	M11 240111	178	M11 240237	221	M11 240403	264	M11 240710	307	M11 241050	350	M12 240273
7	M10 240484	50	M10 240651	93	M11 240031	136	M11 240113	179	M11 240243	222	M11 240405	265	M11 240712	308	M11 241051	351	M12 240274
8	M10 240507	51	M10 240652	94	M11 240032	137	M11 240116	180	M11 240246	223	M11 240412	266	M11 240713	309	M11 241055	352	M12 240277
9	M10 240508	52	M10 240659	95	M11 240034	138	M11 240117	181	M11 240247	224	M11 240420	267	M11 240716	310	M11 241055	353	M12 240284
10	M10 240511	53	M10 240661	96	M11 240035	139	M11 240118	182	M11 240249	225	M11 240422	268	M11 240717	311	M11 241057	354	M12 240287
11	M10 240514	54	M10 240665	97	M11 240036	140	M11 240119	183	M11 240258	226	M11 240424	269	M11 240719	312	M11 241058	355	M12 240288
12	M10 240520	55	M10 240666	98	M11 240037	141	M11 240122	184	M11 240261	227	M11 240425	270	M11 240721	313	M11 241059	356	M12 240289
13	M10 240528	56	M10 240668	99	M11 240038	142	M11 240123	185	M11 240263	228	M11 240427	271	M11 240723	314	M11 241060	357	M12 240290
14	M10 240530	57	M10 240671	100	M11 240039	143	M11 240126	186	M11 240266	229	M11 240428	272	M11 240724	315	M11 241061	358	M12 240291
15	M10 240531	58	M10 240675	101	M11 240040	144	M11 240128	187	M11 240277	230	M11 240430	273	M11 240725	316	M11 241063	359	M12 240293
16	M10 240534	59	M10 240676	102	M11 240041	145	M11 240129	188	M11 240278	231	M11 240434	274	M11 240726	317	M11 241064	360	M12 240294
17	M10 240536	60	M10 240677	103	M11 240042	146	M11 240131	189	M11 240280	232	M11 240435	275	M11 240727	318	M11 241065	361	M12 240296
18	M10 240540	61	M10 240684	104	M11 240043	147	M11 240134	190	M11 240284	233	M11 240436	276	M11 240728	319	M11 241067	362	M12 240299
19	M10 240546	62	M10 240685	105	M11 240044	148	M11 240137	191	M11 240287	234	M11 240437	277	M11 240731	320	M11 241068	363	M12 240300
20	M10 240547	63	M10 240687	106	M11 240045	149	M11 240139	192	M11 240289	235	M11 240440	278	M11 240733	321	M11 241069	364	M12 240301
21	M10 240550	64	M10 240693	107	M11 240046	150	M11 240145	193	M11 240294	236	M11 240441	279	M11 240734	322	M11 241072	365	M12 240302
22	M10 240553	65	M10 240694	108	M11 240047	151	M11 240146	194	M11 240297	237	M11 240442	280	M11 240735	323	M11 241073	366	M12 240303
23	M10 240566	66	M10 240698	109	M11 240048	152	M11 240147	195	M11 240298	238	M11 240443	281	M11 240736	324	M11 241074	367	M12 240305
24	M10 240572	67	M10 240746	110	M11 240050	153	M11 240157	196	M11 240302	239	M11 240445	282	M11 240737	325	M11 241075	368	M12 240306
25	M10 240579	68	M10 240747	111	M11 240052	154	M11 240163	197	M11 240303	240	M11 240446	283	M11 240738	326	M11 241077	369	M12 240307
26	M10 240580	69	M10 240748	112	M11 240053	155	M11 240166	198	M11 240304	241	M11 240447	284	M11 240740	327	M11 241077	370	M12 240308
27	M10 240583	70	M10 240749	113	M11 240054	156	M11 240167	199	M11 240305	242	M11 240448	285	M11 240741	328	M11 241078	371	M12 240309
28	M10 240587	71	M10 240750	114	M11 240055	157	M11 240168	200	M11 240309	243	M11 240450	286	M11 240742	329	M12 240000	372	M12 240310
29	M10 240590	72	M10 240751	115	M11 240056	158	M11 240174	201	M11 240312	244	M11 240451	287	M11 240743	330	M12 240001	373	M12 240314
30	M10 240591	73	M10 240752	116	M11 240057	159	M11 240176	202	M11 240313	245	M11 240453	288	M11 240745	331	M12 240002	374	M12 240315
31	M10 240602	74	M10 240753	117	M11 240058	160	M11 240181	203	M11 240314	246	M11 240453	289	M11 240749	332	M12 240003	375	M12 240317
32	M10 240606	75	M10 240754	118	M11 240059	161	M11 240183	204	M11 240315	247	M11 240456	290	M11 240750	333	M12 240156	376	M12 240318
33	M10 240607	76	M10 240756	119	M11 240060	162	M11 240189	205	M11 240316	248	M11 240457	291	M11 241028	334	M12 240160	377	M12 240319
34	M10 240613	77	M10 240759	120	M11 240061	163	M11 240192	206	M11 240319	249	M11 240458	292	M11 241031	335	M12 240245	378	M12 240320
35	M10 240614	78	M10 240761	121	M11 240062	164	M11 240193	207	M11 240322	250	M11 240459	293	M11 241032	336	M12 240248	379	M12 240321
36	M10 240616	79	M10 240762	122	M11 240063	165	M11 240206	208	M11 240323	251	M11 240461	294	M11 241033	337	M12 240249	380	M12 240322
37	M10 240618	80	M10 240763	123	M11 240064	166	M11 240207	209	M11 240330	252	M11 240463	295	M11 241034	338	M12 240250	381	M12 240323
38	M10 240622	81	M11 240018	124	M11 240065	167	M11 240209	210	M11 240333	253	M11 240465	296	M11 241035	339	M12 240251	382	M12 240324
39	M10 240624	82	M11 240019	125	M11 240066	168	M11 240210	211	M11 240335	254	M11 240466	297	M11 241036	340	M12 240252	383	M12 240325
40	M10 240626	83	M11 240021	126	M11 240067	169	M11 240211	212	M11 240337	255	M11 240467	298	M11 241037	341	M12 240253	384	M12 240326
41	M10 240632	84	M11 240022	127	M11 240068	170	M11 240212	213	M11 240338	256	M11 240473	299	M11 241039	342	M12 240254	385	M12 240328
42	M10 240633	85	M11 240023	128	M11 240069	171	M11 240214	214	M11 240339	257	M11 240475	300	M11 241040	343	M12 240255	386	M12 240329
43	M10 240636	86	M11 240024	129	M11 240070	172	M11 240215	215	M11 240344	258	M11 240485	301	M11 241042	344	M12 240257	387	M12 240330

DNA sequences from meningococcal isolates 1-392 and *N. lactamica* isolates L1-L16 were used to assess primer-site conservation. Shaded meningococcal isolates (393-407) were used to identify new potential primer-sites.

Appendix II: Isolates used for *fHbp* PCR assay optimisation and the determination of the analytical sensitivity

Isolate no.	Isolate ID	Group	ST	CC	<i>fHbp</i> allele	fHbp peptide
1	*M08 240297	B	32	ST-32 complex	1	1
2	M07 240954	C	491	ST-11 complex	10	10
3	M07 241036	B	269	ST-269 complex	15	15
4	*M07 241073	B	162	ST-162 complex	21	12
5	*M07 240725	B	2080	ST-41/44 complex	348	109
6	*M08 240113	B	213	ST-213 complex	30	30
7	M08 240032	B	461	ST-461 complex	71	47

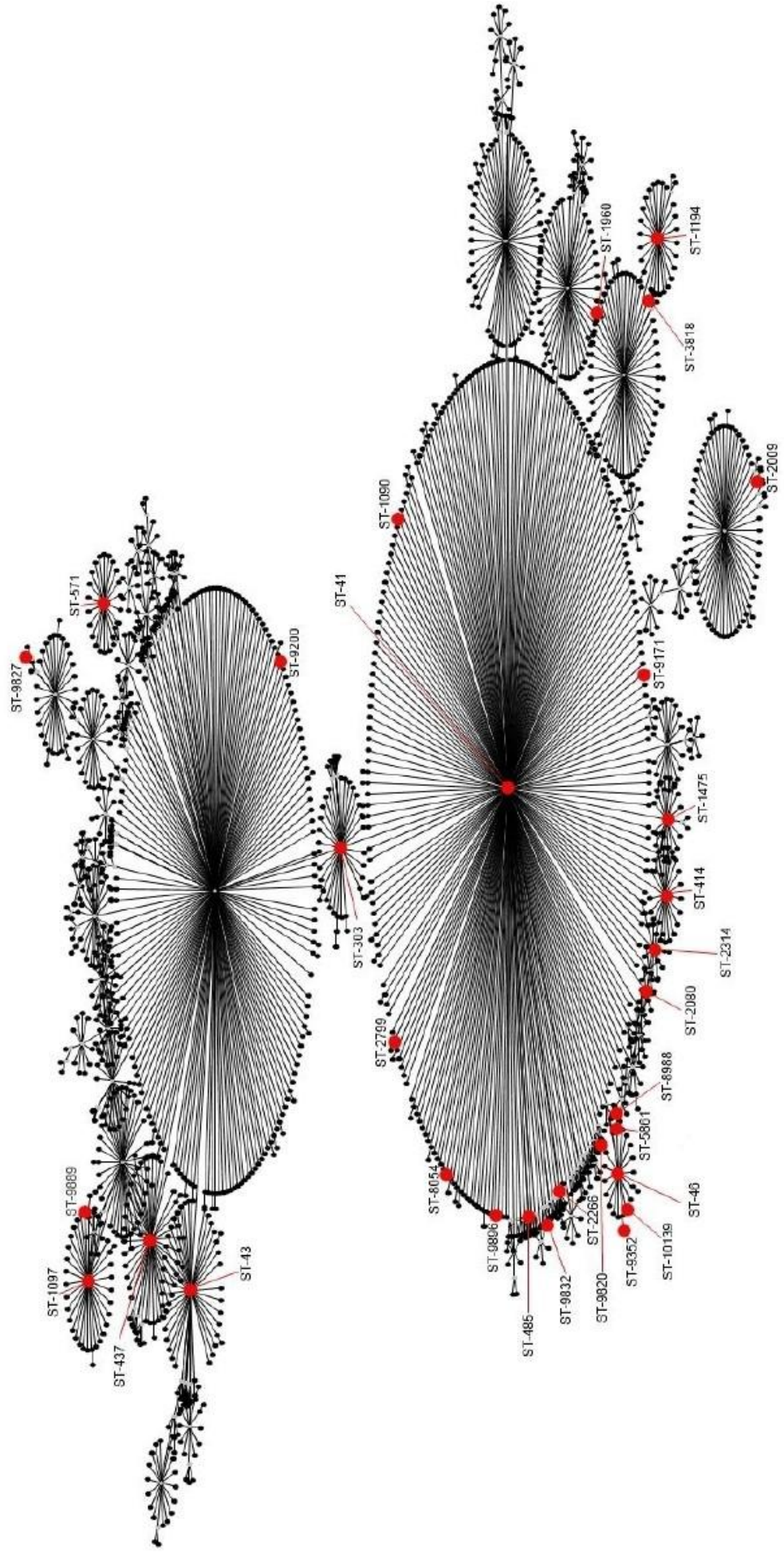
*isolate used for primer optimisation

Appendix III: eBURST and SplitsTree diagrams of *fHbp* PCR assay validation panel strains.

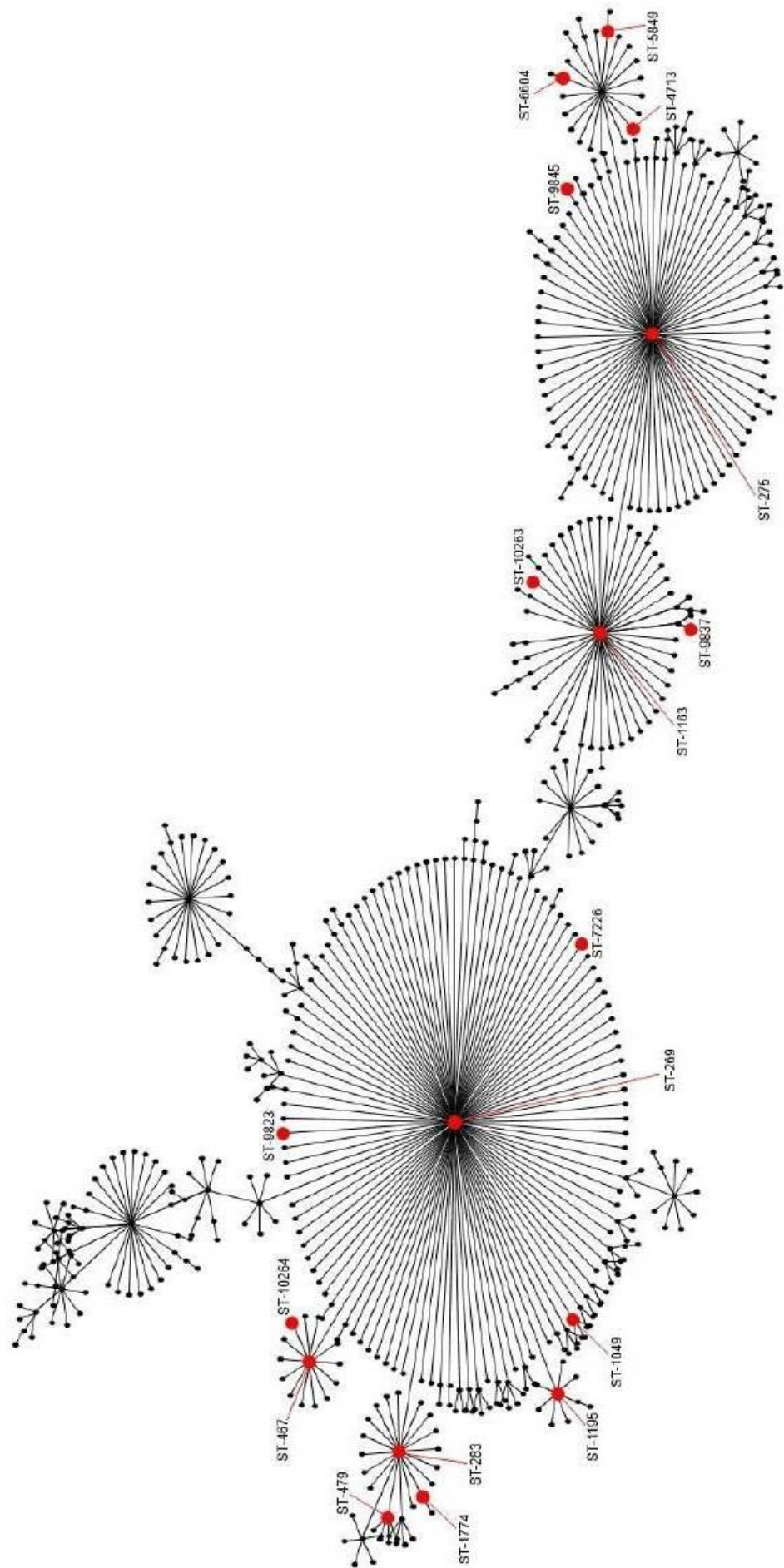
All MLST profile data were downloaded from PubMLST on 30/12/2013. The annotated red dots on the diagrams indicate validation panel isolate STs. The eBURST diagrams represent seven common clonal complexes: A= ST-41/44 complex, B= ST-269 complex, C= ST-32 complex, D= ST-22 and ST-23 complexes, E= ST-213 complex and F= ST-60 complex. According to PubMLST, ST-10281 is part of the ST-213 complex, however, in this analysis, there was no SLV linking ST-10281 to another member of this eBURST group.

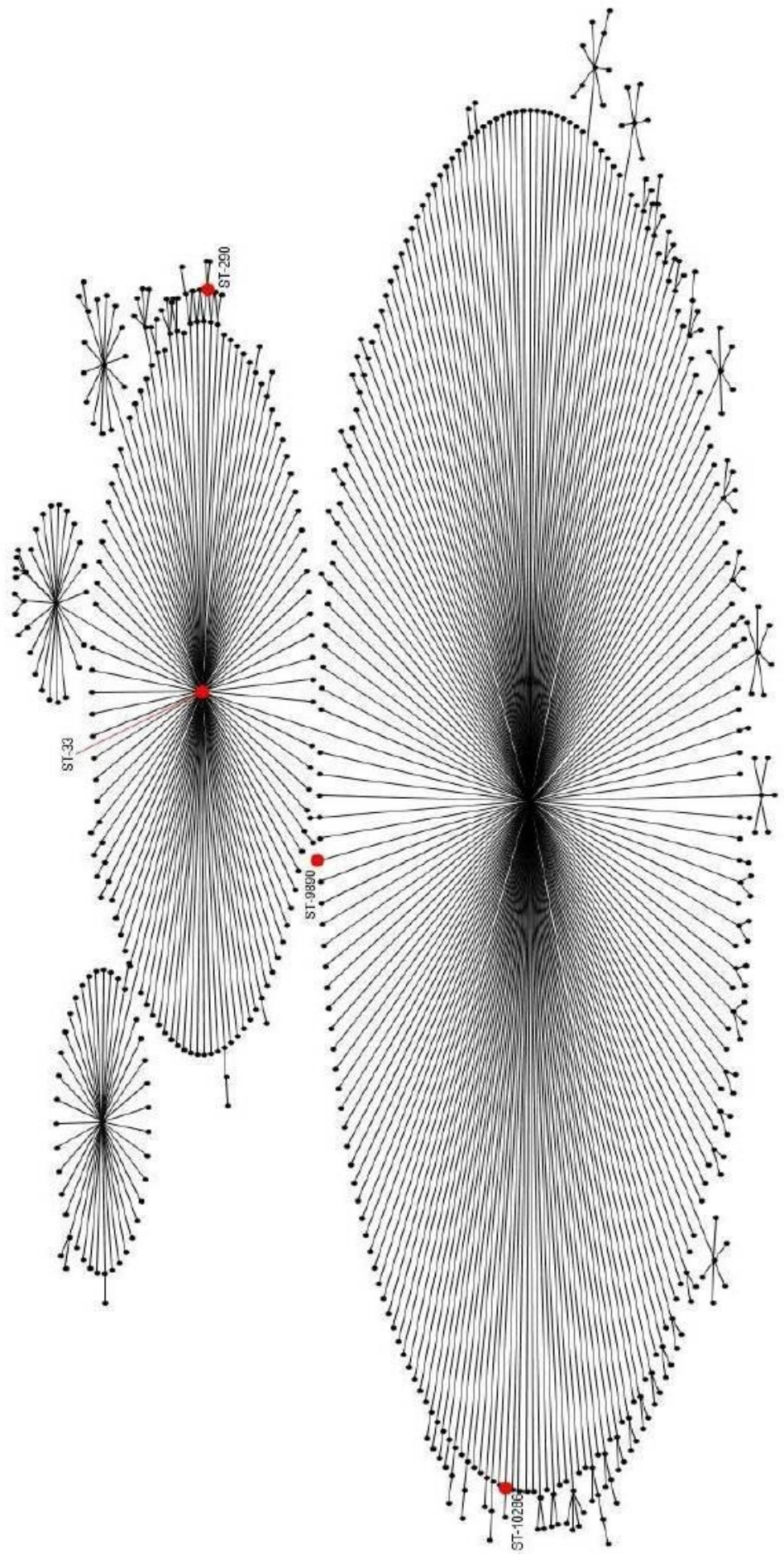
Four of the six ST-11 complex validation panel isolates were of ST-11. Ribosomal MLST analysis was, therefore, performed on ST-11 complex isolate genomes to produce a Neighbor-Net SplitsTree diagram (G). All ST-11 complex isolate genomes within the PubMLST database (n= 177) were analysed and the six validation isolates were highlighted in red on the tree.

A:

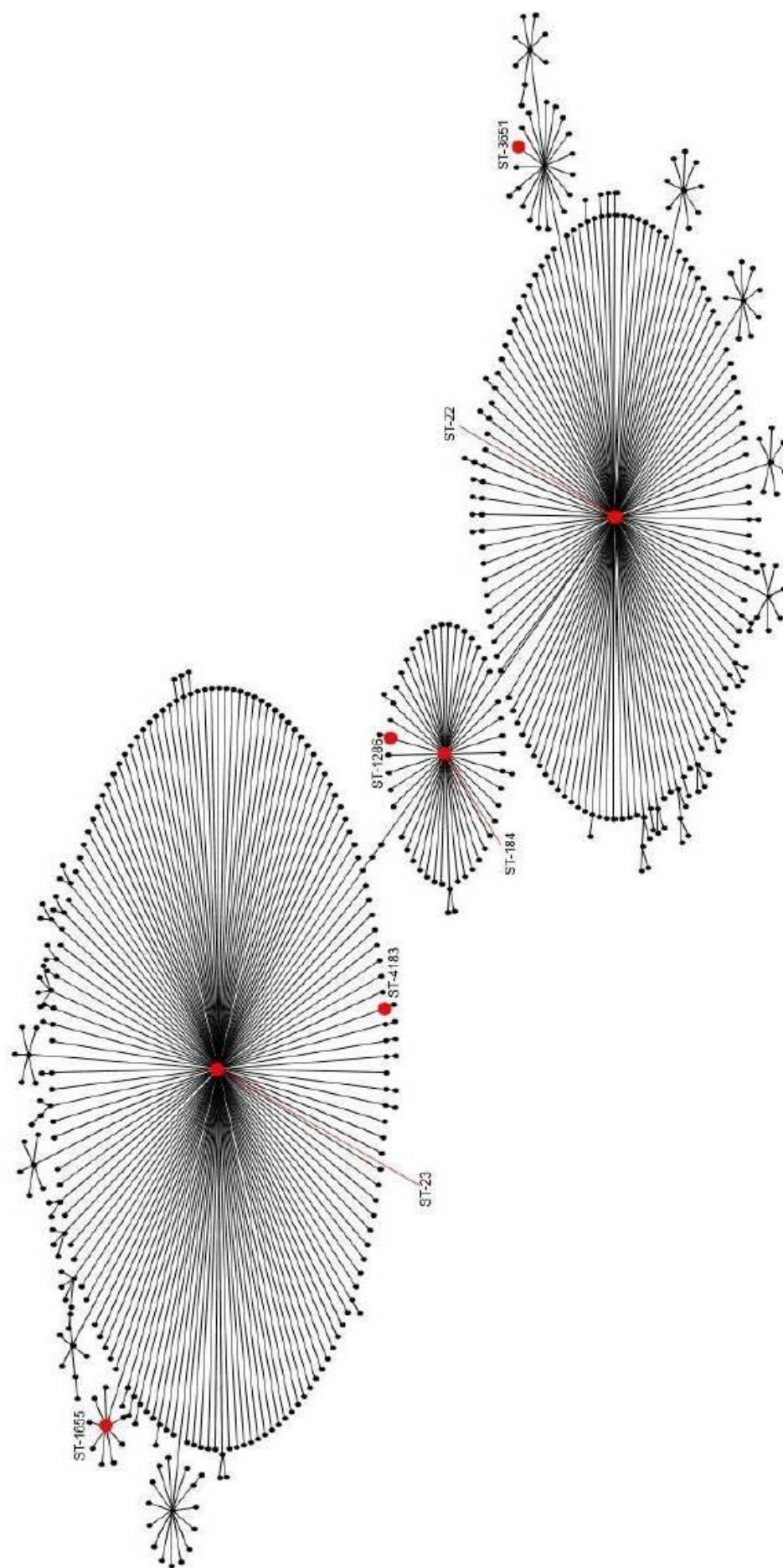


B:

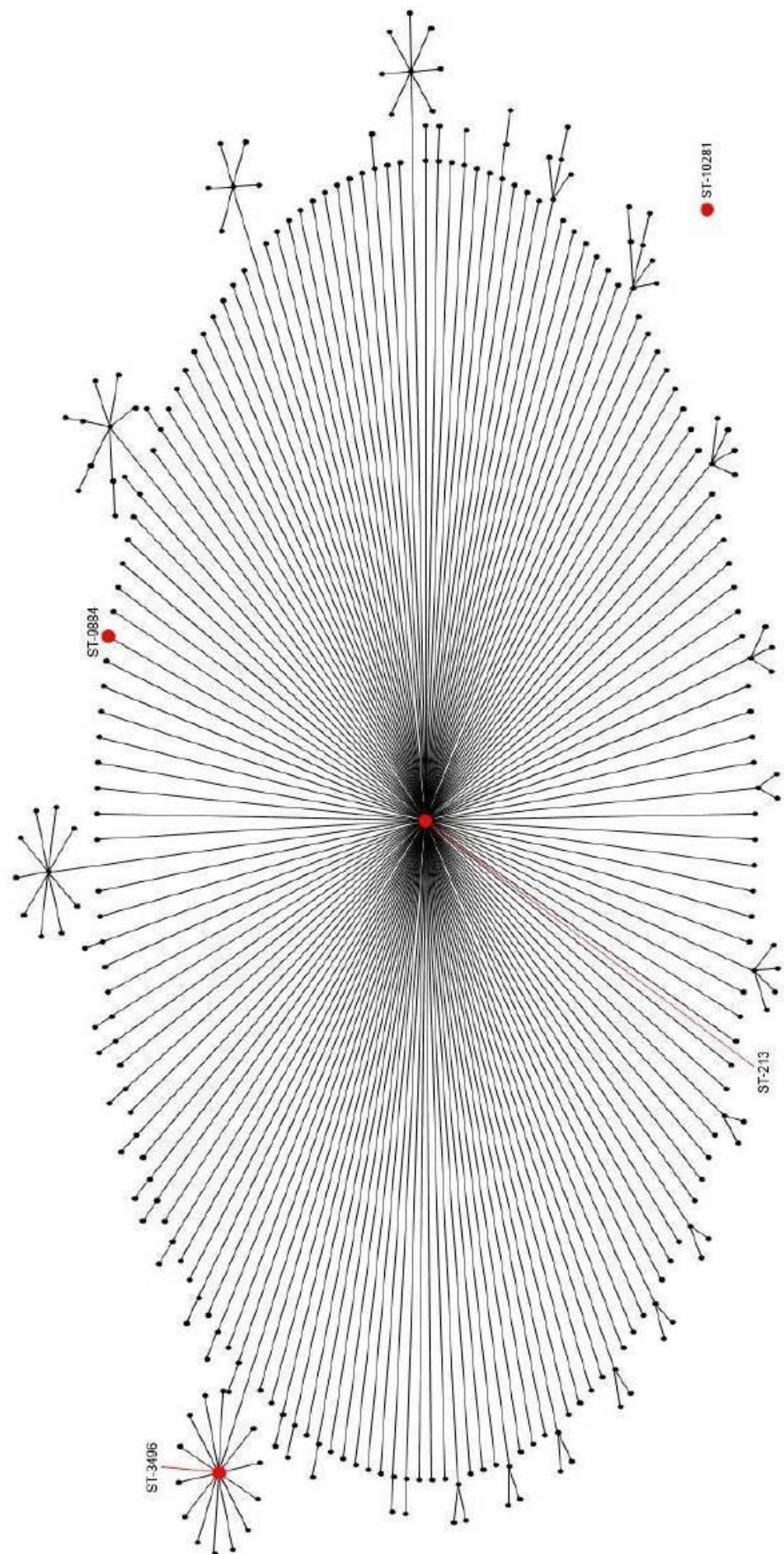




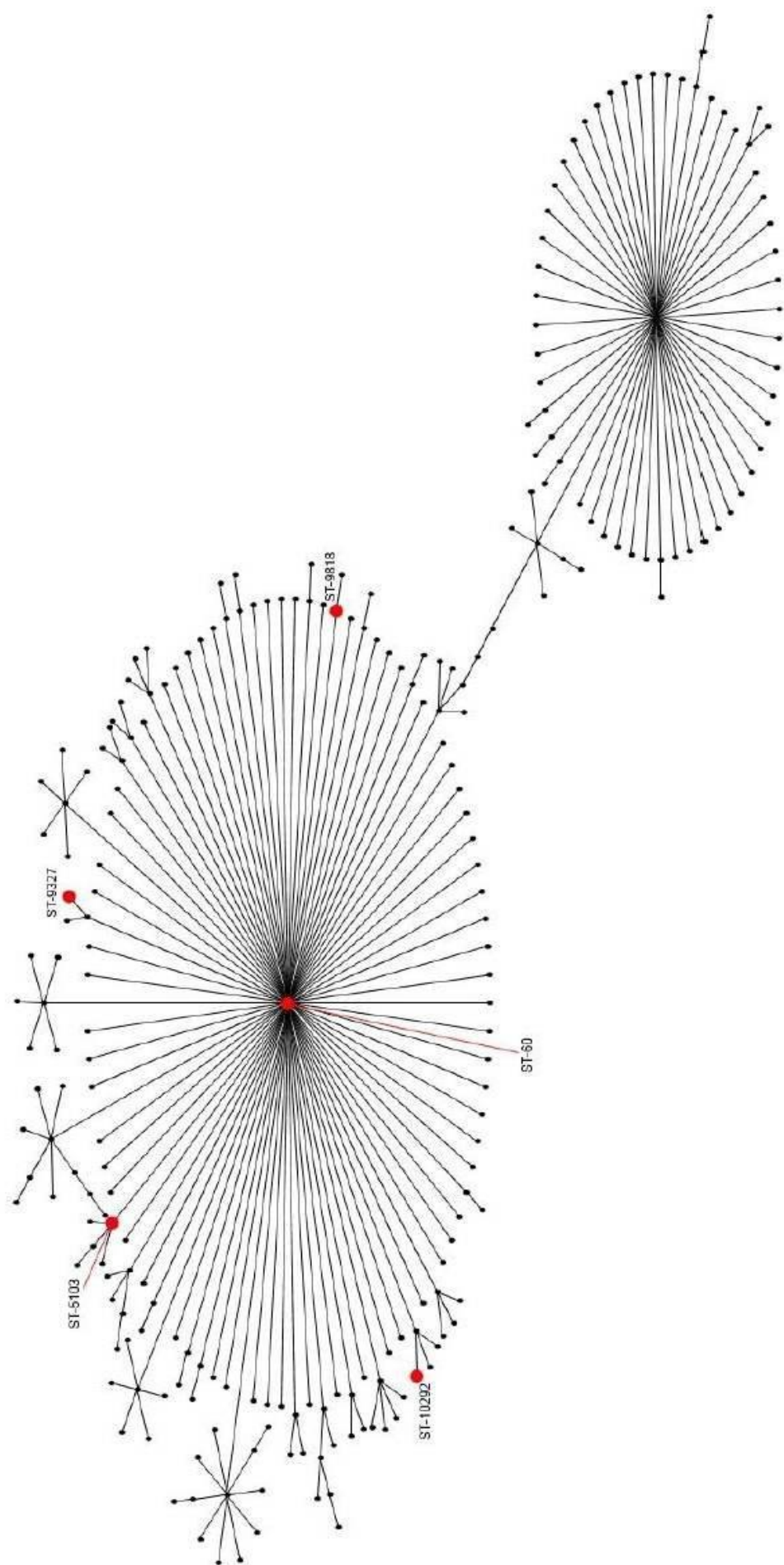
D:



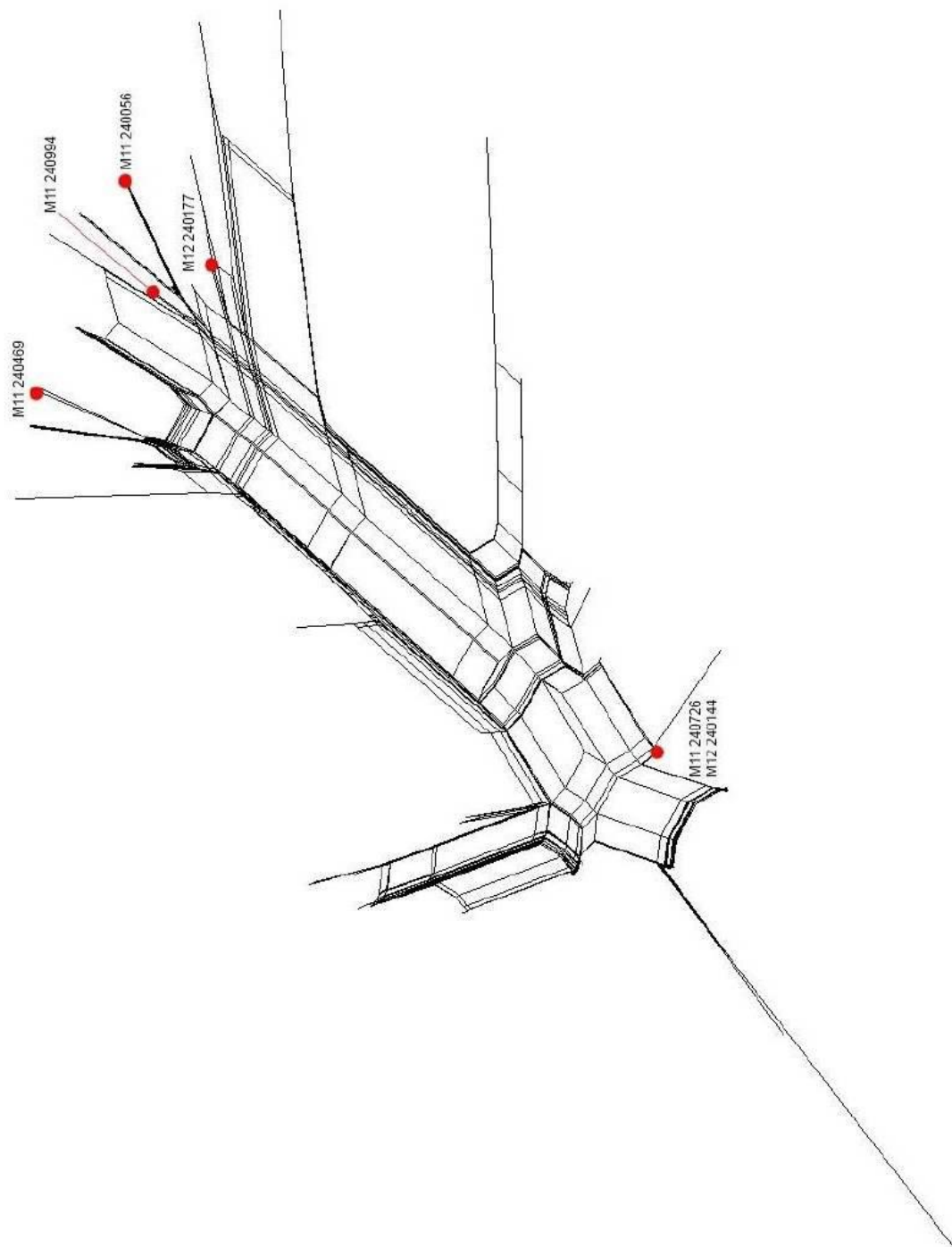
E:



F:



G:



Appendix IV: Validation panel of 96 isolates/clinical specimen pairs
selected to approximate the strain diversity among invasive
meningococci in England and Wales.

Clinical Isolates				Clinical Specimens				Clinical Isolates				Clinical Specimens					
ID	Group	ST	CC	MGL-assigned Hbp allele	ID	Specimen n Type*	Specimen ctrA Ct Value**	Specimen volume (μL) used in extraction	ID	Group	ST	CC	MGL- assigned Hbp allele	ID	Specimen Type*	Specimen ctrA Ct Value**	Specimen volume (μL) used in extraction
M11 240475	B	4051	Singleton	4	M11 942010	EDTA	29	100	M11 240501	B	10263	ST-269 complex	19	M11 945434	CSF	30	50
M11 240723	B	1345	Singleton	14	M11 951518	EDTA	33	100	M11 240593	B	10264	ST-269 complex	15	M11 946553	EDTA	35	100
M11 240002	B	4954	Singleton	14	M11 900874	CSF	44	50	M11 240189	B	8068	ST-282 complex	69	M11 909694	EDTA	26	100
M11 240323	B	9847	Singleton	13	M11 919874	EDTA	34	100	M11 240285	B	33	ST-32 complex	151	M11 916311	EDTA	36	100
M11 240402	B	9825	Singleton	No allele detected	M11 929760	CSF	33	70	M11 240082	B	290	ST-32 complex	1	M11 902644	CLOT	25	100
M11 240234	C	5238	Singleton	1	M11 913431	EDTA	27	100	M11 240013	B	9890	ST-32 complex	593	M11 900970	EDTA	29	75
M12 240194	B	5151	Singleton	1	M12 910062	BLOOD	26	100	M12 240149	B	10286	ST-32 complex	272	M12 904853	BLOOD	29	100
M12 240120	B	10263	Singleton	19	M12 900860	BLOOD	27	100	M11 240742	B	35	ST-35 complex	708	M11 953368	EDTA	34	100
M11 240347	C	5133	ST-103 complex	19	M11 922854	EDTA	34	100	M11 240060	B	2380	ST-35 complex	92	M11 901430	EDTA	27	100
M11 240056	B	11	ST-11 complex	715	M11 900926	CSF	18	100	M11 240103	B	41	ST-41/44 complex	788	M11 963565	EDTA	31	100
M11 240726	W	11	ST-11 complex	715	M11 951934	EDTA	27	100	M12 240131	NG	41	ST-41/44 complex	4	M12 902371	BLOOD	32	100
M11 240994	C	11	ST-11 complex	787	M11 963097	EDTA	38	100	M12 240168	B	43	ST-41/44 complex	19	M12 906589	BLOOD	31	100
M12 240177	C	11	ST-11 complex	361	M12 908623	BLOOD	34	100	M12 240128	B	46	ST-41/44 complex	4	M12 901848	BLOOD	29	100
M11 240469	B	10260	ST-11 complex	100	M11 941014	EDTA	23	100	M11 240295	B	303	ST-41/44 complex	4	M11 917928	CSF	29	100
M12 240144	W	10264	ST-11 complex	22	M12 904562	BLOOD	32	100	M12 240169	NG	414	ST-41/44 complex	19	M12 906176	BLOOD	28	100
M11 240338	B	3737	ST-1157 complex	68	M11 921330	EDTA	26	100	M11 240119	B	437	ST-41/44 complex	30	M11 904062	EDTA	32	100
M11 240126	B	10268	ST-1157 complex	68	M11 964223	EDTA	31	100	M11 240088	B	485	ST-41/44 complex	4	M11 903062	EDTA	31	100
M11 240479	B	162	ST-162 complex	21	M11 941974	CSF	31	100	M12 240236	B	571	ST-41/44 complex	19	M12 921414	CSF	27	50
M11 240045	B	858	ST-18 complex	36	M11 900891	EDTA	23	100	M11 240255	B	1090	ST-41/44 complex	4	M11 915226	EDTA	26	100
M12 240123	B	5529	ST-18 complex	626	M11 964187	EDTA	31	100	M11 240193	B	1097	ST-41/44 complex	562	M11 910517	EDTA	27	100
M12 240145	B	213	ST-213 complex	29	M12 904524	BLOOD	30	100	M11 241044	B	1194	ST-41/44 complex	4	M11 966768	EDTA	28	100
M12 240092	B	3496	ST-213 complex	1	M12 898016	BLOOD	28	100	M11 240195	B	1475	ST-41/44 complex	14	M11 910483	CSF	28	100
M12 240208	B	9884	ST-213 complex	44	M12 911076	BLOOD	27	100	M11 240244	B	1980	ST-41/44 complex	14	M11 913861	EDTA	26	100
M12 240107	B	10281	ST-213 complex	205	M12 898817	BLOOD	26	100	M11 240472	B	2009	ST-41/44 complex	24	M11 940457	EDTA	26	100
M11 240349	W	22	ST-22 complex	69	M11 922561	EDTA	29	100	M11 240145	B	2080	ST-41/44 complex	19	M11 906944	EDTA	37	100
M11 240231	W	184	ST-22 complex	16	M11 912817	EDTA	33	100	M12 240309	B	2266	ST-41/44 complex	14	M12 923425	CSF	24	90
M11 240029	W	1286	ST-22 complex	74	M11 900986	EDTA	28	100	M12 240187	B	2314	ST-41/44 complex	4	M12 909491	BLOOD	36	100
M11 241064	Y	3651	ST-22 complex	25	M11 969988	EDTA	33	100	M11 240097	B	2799	ST-41/44 complex	4	M11 904290	CSF	22	100
M11 240312	Y	23	ST-23 complex	25	M11 918182	SERUM	36	100	M11 240125	B	3818	ST-41/44 complex	4	M11 905224	CSF	33	100
M11 240982	Y	1655	ST-23 complex	25	M11 959902	EDTA	30	100	M11 240034	B	5861	ST-41/44 complex	73	M11 900929	CSF	33	40
M11 240411	Y	4183	ST-23 complex	25	M11 932904	EDTA	28	100	M11 240766	B	8054	ST-41/44 complex	14	M11 954754	EDTA	23	100
M11 240030	B	269	ST-269 complex	15	M11 900480	EDTA	31	100	M11 240506	B	8988	ST-41/44 complex	73	M11 945987	EDTA	18	100
M11 240368	B	269	ST-269 complex	15	M11 925218	EDTA	34	75	M12 240134	B	9171	ST-41/44 complex	4	M12 903101	CSF	29	100
M11 240212	B	275	ST-269 complex	19	M11 912092	EDTA	27	100	M11 240457	B	9200	ST-41/44 complex	356	M11 940244	CSF	28	100
M11 240775	B	283	ST-269 complex	15	M11 955585	EDTA	34	100	M12 240315	B	9352	ST-41/44 complex	14	M12 923786	BLOOD	37	100
M12 240323	C	467	ST-269 complex	15	M12 925366	BLOOD	33	100	M11 240018	B	9820	ST-41/44 complex	4	M11 900925	EDTA	26	100
M11 240751	B	479	ST-269 complex	15	M11 906706	CSF	20	100	M11 240431	B	9827	ST-41/44 complex	19	M11 934622	EDTA	23	100
M11 240785	B	1049	ST-269 complex	717	M11 909484	CSF	24	100	M11 240059	B	9832	ST-41/44 complex	4	M11 902886	EDTA	32	100
M11 240484	B	1163	ST-269 complex	235	M11 943211	EDTA	33	100	M11 240728	B	9889	ST-41/44 complex	713	M11 951931	EDTA	27	100
M12 240116	B	1195	ST-269 complex	15	M12 901115	CSF	42	95	M11 240311	B	9896	ST-41/44 complex	4	M11 919007	EDTA	30	100
M11 240086	B	1774	ST-269 complex	149	M11 902822	EDTA	26	100	M11 240170	B	10139	ST-41/44 complex	281	M11 906901	EDTA	22	100
M12 240216	B	4713	ST-269 complex	13	M12 911596	BLOOD	33	100	M11 240988	B	461	ST-461 complex	71	M11 962849	EDTA	23	100
M12 240303	B	5849	ST-269 complex	13	M12 921916	BLOOD	27	100	M11 240262	A	4789	ST-5 complex	719	M11 915746	EDTA	30	100
M11 240334	B	6004	ST-269 complex	13	M11 920780	EDTA	28	100	M11 240404	B	60	ST-60 complex	13	M11 936564	BLOOD	29	100
M11 240993	B	7226	ST-269 complex	15	M11 962537	EDTA	28	100	M11 240394	B	5103	ST-60 complex	13	M11 928895	EDTA	24	80
M11 240388	B	9823	ST-269 complex	15	M11 927727	EDTA	25	100	M11 240167	B	9327	ST-60 complex	13	M11 907243	EDTA	25	100
M11 240309	B	9837	ST-269 complex	19	M11 919141	EDTA	31	100	M10 240825	B	9818	ST-60 complex	13	M11 900880	SERUM	39	100
M11 240243	B	9845	ST-269 complex	13	M11 913874	EDTA	30	100	M12 240222	B	10292	ST-60 complex	13	M12 912364	BLOOD	28	100

*EDTA- Ethylenediaminetetraacetic Acid-Treated Blood Sample, BLOOD- Blood Sample, CSF- Cerebrospinal Fluid Sample, SERUM- Serum Sample, CLOT- Clotted Blood Sample.

Appendix V: *fHbp* genotyping results for validation panel of 96 isolates/clinical specimen pairs.

[illegible]

Appendix VI: Discrepant NEIS alleles among the specimen and isolate.

Locus	Product*	Allele in M15 901648	Allele in M15 240178	No. of nucleotide differences	Genbank BLAST result	Comments
NEIS0835	hypothetical protein	223	20	1	ND	n/a
NEIS1901 (lgtB)	lacto-N-neotetraose biosynthesis glycosyl transferase	200	359	1	ND	n/a
NEIS2580	hypothetical protein	20	166	1	ND	n/a
NEIS2854	D12 class N6 adenine-specific DNA methyltransferase	8	1	1	ND	n/a
NEIS0591	hypothetical protein	2	7	2	Partial additional hits (~230bp) in most Genbank genomes.	n/a
NEIS0802	hypothetical protein	78	5	5	Paralogous in three Genbank genomes (alpha522, H44/76 and M01 240355).	n/a
NEIS1452	hypothetical protein	1056	17	30 (indel)	Partial additional hits (~100bp) in all Genbank genomes.	Repeated motif. Probable assembly artefact.
NEIS1900 (lgtE)	lacto-N-neotetraose biosynthesis glycosyl transferase	294	57	249	Two significant hits in all Genbank genomes. Homologous to NEIS 1901 (lgtB).	n/a
Locus	Product*	Allele in M15 908607	Allele in M15 240270	No. of nucleotide differences	Genbank BLAST result	Comments
NEIS0953	hypothetical protein	55	22	3	Paralog of NEIS1664. Two-three full hits in all Genbank genomes.	n/a
NEIS1664	hypothetical protein	55	22	3	Paralog of NEIS0953. Two-three full hits in all Genbank genomes.	n/a
NEIS0534 (rpsP)	30S ribosomal protein S16	4	22	4	Single hits in all Genbank genomes. No evidence of paralogy.	Encodes ribosomal subunit. Highly conserved.
NEIS1940	hypothetical protein	5	6	4	Paralogous in one Genbank genome (DE10444).	n/a
Locus	Product*	Allele in M15 894870	Allele in M15 240103	No. of nucleotide differences	Genbank BLAST result	Comments
NEIS0361	hypothetical protein	127	79;102	1	ND	Two identified alleles in isolate genome. Possibly paralogous
NEIS0957	hypothetical protein	23	15	1	ND	n/a
NEIS1792	hypothetical protein	3	4	2	Two hits in one Genbank genome (alpha14)	n/a
NEIS1958	hypothetical protein	5	139	9	Paralogous in four Genbank genomes plus small (115bp) matches in all.	n/a
Locus	Product*	Allele in M15 897440	Allele in M15 240142	No. of nucleotide differences	Genbank BLAST result	Comments
NEIS0164	valyl-tRNA synthetase	1072	3	1	ND	n/a
NEIS0276	hypothetical protein	95	21	1	ND	n/a
NEIS0581 (galU)	glucose 1-phosphate uridylyltransferase	420	10	1	ND	n/a
NEIS0718	signal peptidase I	299	4	1	ND	n/a
NEIS1702	hypothetical protein	18	60	1	ND	n/a
NEIS0036 (pilT1)	type IV pilus retraction ATPase PilT	562	14	2	Single hits in all Genbank genomes. No evidence of paralogy.	n/a
NEIS2905	hypothetical protein	277	271	2	Partial additional hits (~100bp) in all Genbank genomes.	n/a
NEIS2099	putative immunity protein	5	19	5	Paralogous in several Genbank genomes including H44/76 and MC58 (cc32).	n/a
NEIS2451	hypothetical protein	4	1	22	Paralog of NEIS2461. Multiple hits in several Genbank genomes.	n/a
NEIS2461	hypothetical protein	1	4	22	Paralog of NEIS2451. Multiple hits in several Genbank genomes.	n/a
NEIS1865	hypothetical protein	180	142	34 with indel	Paralog of NEIS0027. Multiple hits in several Genbank genomes.	n/a
Locus	Product*	Allele in M15 948231	Allele in M15 240805	No. of nucleotide differences	Genbank BLAST result	Comments
NEIS0832	hypothetical protein	102	113	1	ND	n/a
NEIS1020	hypothetical protein	1	10	1	ND	n/a
NEIS1418	putative membrane peptidase	97	70	1	ND	n/a
NEIS0453	hypothetical protein	67	5	5	Paralogous. Two full-sizes hits in many Genbank genomes including closest ref genome (M01 240355)	n/a
NEIS0213 (pglA)	pilin glycosyltransferase	321	305	2 (indel)	Single hits in all Genbank genomes. No evidence of paralogy.	Poly-G tract. Probable PCR error.
NEIS1452	hypothetical protein	892	39	75 (indel)	Partial additional hits (~100bp) in all Genbank genomes.	Repeated motif. Probable assembly artefact.
NEIS2409	hypothetical protein	30;52	62;65	up to 11	Paralogous. Two full-sizes hits in seven Genbank genomes including closest ref genome (M01 240355)	n/a
NEIS1214	transcription-repair coupling factor	166	985	234 (indel)	Multiple partial additional hits in most Genbank genomes.	n/a
Locus	Product*	Allele in M15 933165	Allele in M15 240650	No. of nucleotide differences	Genbank BLAST result	Comments
NEIS0046 (rfbA)	glucose-1-phosphate thymidyltransferase	38	148	1	ND	n/a
NEIS1702	hypothetical protein	18	60	1	ND	n/a
Locus	Product*	Allele in M15 906731	Allele in M15 240240	No. of nucleotide differences	Genbank BLAST result	Comments
NEIS1598	chorismate synthase	116	384	1	ND	n/a
NEIS2580	hypothetical protein	59	68	1	ND	n/a
NEIS0276	putative rotamase	193	315	9 (indel)	Small additional hits (189bp) in some Genbank genomes.	Repeated motif. Probable assembly artefact.
Locus	Product*	Allele in M15 901404	Allele in M15 240180	No. of nucleotide differences	Genbank BLAST result	Comments
NEIS0966	phage-related protein	12	9	1	ND	n/a
NEIS2789	hypothetical protein	40	41	1	ND	n/a
NEIS0967	amidase	61;71	187	28 (187 vs 71) and 8 (187 vs 61)	Two alleles assigned for specimen. Paralogous in several Genbank genomes.	n/a

* as described on PubMLST

ND= Only single difference. Genbank BLAST not performed